MAY 2 8 1929

NUMBER 5

OLOGY

OF AMERICAN

PEPTON

Perfectly serviceable for the formulas and in all the technic of the bacteriological and antitoxin laboratory. It is employed in the usual proportions and for whatever purposes Pepton of this most desirable quality is required.

Manufactured by

Fairchild Bros. & Foster

New York

RARE SUGARS

OF BACTERIOLOGICAL IMPORTANCE

Adonitol (Adonite)
l-Arabinose
l-Arabitol (Arabite)
Cellose
Dulcitol (Dulcite)
Erythritol (Erythrite)
d-Galactose
d-Glucose (anhydrous)
d-Glucose (hydrate)
Glycogen
i-Inositol (Inosite)
Inulin
Invert Sugar
Lactose
d-Levulose from Invert Sugar
Maltose (hydrate)
d-Mannitol (Mannite)
d-Mannose
[illegible]
[illegible] (Raffinose) (hydrate)
[illegible] (hydrate)
[illegible] (Sorbite)
[illegible] Soluble (Barfoed-Lintner method)
[illegible]
[illegible]
[illegible]
[illegible]

Reference for determinations supplied

Carried by the leading Distributors

Write for our list of Organic Acids, Laboratory Reagents and Fine Chemicals

Originally Standard of Pharmacy

PFANSTIEHL CHEMICAL COMPANY

160 Market Street [illegible], Illinois

Entered as second-class matter April 6, 1911, at the postoffice at [illegible], under the act of March 3, 1879. [illegible] Act of [illegible], 1917, authorized on [illegible]

THIRTY STRAINS OF GRAM-POSITIVE COCCI ISOLATED FROM CASES OF GENITO-URINARY INFECTIONS

ASYA M. S. STADNICHENKO

Research Laboratory, Municipal Tuberculosis Sanitarium, Chicago, Ill.

Received for publication, October 19, 1928

Thirty strains of Gram-positive cocci were isolated from 30 cases of genito-urinary infections. All the specimens were collected by the method routinely employed in the Brady Urological Institute,[1] that is, the urinary meatus was cleansed with alcohol, and the anterior urethra by injection of 1:500 per cent Meroxyl. The first urine was allowed to escape, and a specimen was then obtained in a sterile glass tube, the cotton stopper of which was wrapped in paper. In the laboratory specimens were transferred into sterile centrifuge tubes by means of sterile pipettes, and were centrifuged for three to five minutes at 2200 revolutions a minute. After decanting the supernatant urine, direct smears were made and the sediment cultured.

Since coccal urethral contamination is a frequent occurrence, great care was taken not to include in this series any organisms of such origin. All the strains were isolated from cases in which direct smears showed both Gram-positive cocci and leukocytes. Also, the bacteriological findings were correlated with the clinical histories,[2] and no case of known possible contamination was included.

All the strains came from cases of a single infection (Gram-positive cocci only), except two cases, where direct smears showed

[1] The experiments serving as the basis of this paper were carried out by the author while working in the Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Maryland.

[2] It is a pleasant duty to acknowledge my great indebtedness to Dr. J. A. C. Colston with whose help it was possible to make these correlations. Also I wish to express my gratitude to Justina H. Hill for her interest in this work.

PEPTON

Perfectly serviceable for the formulas and in all the technic of the bacteriological and antitoxin laboratory. It is employed in the usual proportions and for whatever purposes Pepton of this most desirable quality is required.

Manufactured by

Fairchild Bros. & Foster

New York

RARE SUGARS

OF BACTERIOLOGICAL IMPORTANCE

Adonitol (Adonite)
l-Arabinose
l-Arabitol (Arabite)
Cellose
Dulcitol (Dulcite)
Erythritol (Erythrite)
d-Galactose
d-Glucose (anhydrous)
d-Glucose (hydrate)
Glycogen
i-Inositol (Inosite)
Inulin
Invert Sugar
Lactose
d-Levulose from Invert Sugar
Maltose (hydrate)
d-Mannitol (Mannite)
d-Mannose
[illegible]
[illegible] (Raffinose)
[illegible] (Saccharose)
[illegible] (Sucrose)
[illegible]
[illegible] (Trehalose)
[illegible]
[illegible]

d-Glucose for Fermentation Tube Work

Carried by the leading Distributors

Write for our list of Amino Acids, Laboratory Reagents and Rare Chemicals

Specify Standard of Purity Desired

PFANSTIEHL CHEMICAL CO.

160 Market Street

Entered as second-class matter April 11, 1916, at the postoffice at [illegible] under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section [illegible] Act of October 3, 1917, authorized on June 4, 1920.

Copyright, 1920, The Williams & Wilkins Co.

THIRTY STRAINS OF GRAM-POSITIVE COCCI ISOLATED FROM CASES OF GENITO-URINARY INFECTIONS

ASYA M. S. STADNICHENKO

Research Laboratory, Municipal Tuberculosis Sanitarium, Chicago, Ill.

Received for publication, October 19, 1928

Thirty strains of Gram-positive cocci were isolated from 30 cases of genito-urinary infections. All the specimens were collected by the method routinely employed in the Brady Urological Institute,[1] that is, the urinary meatus was cleansed with alcohol, and the anterior urethra by injection of 1:500 per cent Meroxyl. The first urine was allowed to escape, and a specimen was then obtained in a sterile glass tube, the cotton stopper of which was wrapped in paper. In the laboratory specimens were transferred into sterile centrifuge tubes by means of sterile pipettes, and were centrifuged for three to five minutes at 2200 revolutions a minute. After decanting the supernatant urine, direct smears were made and the sediment cultured.

Since coccal urethral contamination is a frequent occurrence, great care was taken not to include in this series any organisms of such origin. All the strains were isolated from cases in which direct smears showed both Gram-positive cocci and leukocytes. Also, the bacteriological findings were correlated with the clinical histories,[2] and no case of known possible contamination was included.

All the strains came from cases of a single infection (Gram-positive cocci only), except two cases, where direct smears showed

[1] The experiments serving as the basis of this paper were carried out by the author while working in the Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Maryland.

[2] It is a pleasant duty to acknowledge my great indebtedness to Dr. J. A. C. Colston with whose help it was possible to make these correlations. Also I wish to express my gratitude to Justina H. Hill for her interest in this work.

both Gram-positive cocci and Gram-negative bacilli. In these two instances special care was taken to isolate the cocci in pure cultures. Later, one of these cases proved to be a fatal case of *Staphylococcus aureus* septicemia. Staphylococci isolated from the patient's blood were found to be identical with the organism that came from his bladder.

In most cases organisms were isolated from the bladder only, but in cases where the kidneys were involved also, and a catheterized specimen obtained, a comparative study was made. Invariably, organisms from the same host gave the same reactions in all the tests employed.

All the strains were isolated by fishing a single colony from twenty-four lactose-agar plates. Sub-culturing of the stock cultures was done every five or six weeks. Some of the cultures were in my possession for over two years, during which period they were cultured and re-cultured scores of times. At the end I was not able to find any variation in their cultural characteristics, except one case of the liquefaction of gelatin, which will be discussed later.

GRAM-STAIN

Nicolle's one-minute stain was used:

{1 cc. saturated alcoholic solution of gentian violet.
{9 cc. 2.5 per cent aqueous solution of phenol.
Stain 20 seconds and pour off.
Stain Gram's iodine 20 seconds.
Decolorize with solution 3 parts of alcohol and 1 part of acetone.
Counterstain with Carbol-fuchsin 1 part and 19 parts of H_2O.

The organisms were stained at different times. Invariably, all of them up to a month old retained gentian violet; but with greater age they retained gentian violet only in spots. Such cultures when transferred to a new medium, always re-gained their ability to retain the stain.

CHROMOGENESIS

Chromgenesis was determined by the method described by Winslow and Winslow (1908). Cultures were grown on agar

slants for fourteen days at room temperature (20°C.), and in the incubator (37°C.). Matching was done against the Winslow color chart. Fourteen strains were matched against different hues of orange, one strain gave a typical citron color, and fifteen were white. All the strains showed pigment production within eighteen to thirty-six hours. No new appearance of pigment was observed after thirty-six hours, but in many instances the pigment became more pronounced with age. It seems that the pigment production was better at room temperature than in the incubator, but the difference was not very marked.

All the strains were also grown on Loeffler's serum and potato media. The pigment production on Loeffler's serum seemed more intense than on agar, and this difference was especially noticeable in the first eighteen to forty hours. Potato medium in most cases confirmed the observations made on agar and Loeffler's serum media. One strain, however, while growing well on Loeffler's and agar, failed to grow on potato, in spite of the fact that several transfers were made. It was a white coccus that did not liquefy gelatin, or reduce nitrates; it produced acid in glucose, lactose, sucrose, maltose and mannitol; it decomposed urea, and was classified as *M. ureae*. Two other strains of the same species in this series produced a good growth on potato medium.

GELATIN

Gelatin liquefaction was always tested with a freshly isolated organism. In the course of study this test was repeated on some of the strains two and three times, and, finally, just before the work was completed it was repeated on the whole series. On all the repeated tests, all the organisms gave the same reaction as when first isolated. There was one exception to this, however; one strain (*S. albus*), that was in my possession for two years, at first liquefied gelatin, but later lost that power, though all of its other cultural characteristics remained constant. It was possible later to re-isolate this coccus from the patient's urine. This freshly isolated organism liquefied gelatin, as before. Therefore this particular strain conforms to Kruse's (1896) observation,

that organisms may lose their ability to liquefy gelatin when growing on artificial media for some time. However, the fact that only one strain in this series lost its ability to liquefy gelatin, while a few others that were in my possession as long continued to liquefy it, suggests that different organisms are affected differently by growth on artificial media.

Organisms were grown in gelatin for thirty days at room temperature (20°C.) and in the incubator (37°C.). At the end of this period the set that was growing in the incubator was put into the ice box for one hour. No difference was observed between these two sets. The percentage of liquefiers was higher in the orange group, 9 strains (64 per cent) out of 14 of these liquefying gelatin, while in the white group 6 strains (40 per cent) out of 15 possessed this power. One strain of citron coccus also liquefied gelatin.

MILK

Organisms were grown in litmus milk for fourteen days at 37°C. The orange cocci exhibited less variation in their reactions than the white ones, but the only strain that gave an alkaline reaction in this series belonged to the orange group.

Table 1 gives reactions in litmus milk.

CARBOHYDRATES

The following carbohydrates were used in this series: glucose, sucrose, lactose, maltose, salicin, mannitol, glycerol, dulcitol, dextrin, raffinose and inulin. The medium was a beef-infusion agar made sugar-free, containing 1 per cent of the carbohydrate and double indicators (brom-cresol purple with cresol-red); the reaction was adjusted carefully in accordance with the procedure prescribed in the Manual of Pure Culture Study (1924). Since the double indicators proved to be very sensitive in registering even a slight change from a neutral point, no attempt was made to read pH value. All the strains in this series which fermented any carbohydrates fermented them so strongly that there was no doubt as to the nature of the reaction.

Inoculation were made both along the slant and deep into the

butt. Cultures were always incubated for ten days before being discarded as negative. However, all the carbohydrates that were fermented in this series were fermented within three days. There was only acid; no gas production.

Table 2 gives reactions with the carbohydrates studied.

TABLE 1

Action on milk

MILK	NUMBER	ORANGE	CITRON	WHITE
No change	1	0	0	1
Acidity: Not sufficient for coagulation	4	2	0	2
Acidity: Sufficient for coagulation	22	11	1	10
Peptonization	2	0	0	2
Alkalinity	1	1	0	0
Total	30	14	1	15

TABLE 2

Fermentation of carbohydrates

CARBOHYDRATES	NUMBER	ACID	ORANGE	CITRON	WHITE
Glucose	30	30	14	1	15
Sucrose	30	30	14	1	15
Maltose	30	28	14	1	13
Lactose	30	28	13	1	14
Mannitol	30	27	14	1	12
Glycerol	30	26	14	0	12
Salicin	30	9	2	0	7
Dextrin	30	3	0	0	3
Dulcitol	30	2	0	0	2
Raffinose	30	2	0	0	2
Inulin	30	1	0	0	1

Winslow, Rothberg and Parsons (1920) observed that the strains isolated from pathogenic conditions have a marked tendency to ferment carbohydrates rather strongly. Strains in my series exhibited the same tendency. As table 2 shows, all the strains (100 per cent) fermented glucose and sucrose, and most of them (94 per cent) fermented maltose and lactose; mannitol (90 per cent); and glycerol (87 per cent). The fermentation of mannitol in this series accords with Dudgeon's (1908) observa-

tion, that mannitol is acidified by most of staphylococci obtained from pathogenic sources. Salicin was fermented by 9 strains only, and of these 9 strains 7 were white cocci. Dextrin, dulcitol, raffinose and inulin were fermented by only a few white strains,—none of the orange strains fermented the above mentioned carbohydrates. In the fermentation of carbohydrates, as in milk, the white strains exhibited more variation in their reactions than the orange strains.

UREA

Pasteur (1860) was the first to notice a "spontaneous" fermentation of urine. In (1862) he had proved that this fermentation of urine was produced by a white micrococcus, which was capable of breaking down urea to ammonium carbonate. Miquel (1888) in his laboratory had grown *urococcus* (as he named the coccus capable of fermenting urea), and studied carefully its property in regard to urea fermentation. An organism, from decomposing urine, was described by Flügge (1896), as a white micrococcus generally found in the human or animal body. Flügge recorded that his organisms differs from Pasteur's in its ability to liquefy gelatin, and proposed to name it *M. ureae-liquefaciens*. Hucker (1924) in describing *M. ureae* states that it is a primary soil form, the fundamental characteristic of which is ability to utilize urea.

In my series all the strains have been grown in plain broth, containing 2 per cent urea and phenolphthalein as an indicator. The medium was adjusted to acidity. With the decomposition of urea and the production of alkalinity, there developed a pink color and a strong ammoniacal odor. Cultures were incubated for ten days before being discarded as negative. The strains in this series that were capable of decomposing urea did so within eighteen to forty-eight hours.

In this series, 14 strains decomposed urea; 8 of them were orange strains and 6, white. It appears, therefore, that there is no correlation between pigmentation and urea utilization; nor do any cultural characteristics appear to be common to all these 14 strains, save fermentation of glucose and sucrose. Most (10) of the urea-decomposing strains produced sufficient acid to curdle

milk. The high percentage of urea-positive strains in this series may have some connection with the source of the specimens (urine), but the data in hand are not sufficient to justify a positive statement.

INDOL

Bayne-Jones and Zinninger (1921) gave us, perhaps, the best survey of the literature on the subject of indol production by staphylococci. Their own intensive work in this line confirms the findings of most investigators, viz., they were not able to demonstrate indol-positive staphylococci. In my series all the strains were cultured in 1 per cent peptone and 0.5 per cent K_2HPO_4. Tests for indol production were made by the para-dimethyl-amido-benzald-benzaldehyde method after the second, fourth, and seventh day of incubation at 37°C. All the cultures were indol-negative.

NITRATES

Cultures were grown in nitrate broth, and nitrate reduction was tested with sulphanilic acid and *a*-naphthalamine after the second, fourth, and seventh day of incubation at 37°C. The orange cocci gave a higher percentage of nitrate-reducing strains than the white cocci. Nine (64 per cent) out of 14 orange strains reduced nitrates, while in the white group only 8 (55 per cent) out of 15 possessed this power. The only citron strain in this series also reduced nitrates.

The length of incubation proved of no significance in this test. There was no difference in results of these three sets.

HEMOLYSIN

Neisser and Wechsberg (1901) and Kutscher and Konrich (1904) showed that staphylococci (*S. albus* and *S. aureus*) are capable of producing hemolysin, but they were not able to find a purely saprophytic form with this characteristic. On the other hand Julianelle (1922) found that all of his strains, both parasitic and saprophytic, were capable of producing hemolysin. Hucker (1924) found only a certain percentage of hemolysin-positive strains in his large series. From his work it appeared that the

white strains gave a larger percentage of hemolysin-positive strains than any other group of cocci (orange or yellow; he found none among red cocci). Mellon and Caldwell (1926) reported most of their strains hemolysin-positive.

In this series Brown's (1919) method for making blood plates was employed. Reading was done after twenty-four and forty-eight hours; no further observation was made. Out of 30 strains 6 gave positive reactions; 5 of them were white strains, and only one orange. Hemolysin in all 6 cases was of the *beta* type. There were no other cultural characteristics in common (save fermentation of glucose and sucrose), nor could any special importance be attached to these six strains from a clinical point of view.

CAPSULES

Strains were grown on milk-agar for eighteen hours, and then were examined with India-ink for capsule formation. No definite capsules were observed in any of these strains.

MOTILITY

Strains were grown in peptone broth and, after twelve, eighteen and twenty-four hours of incubation at 37°C., were examined for motility. A little more than a self-rotation movement was observed in some of the cultures, but none exhibited a typical rapid movement across the stage. The length of incubation made no difference.

CLASSIFICATION OF THE STRAINS

The bacteriological findings resulting from the foregoing experiments served as basis in classifying strains in this series. I insert specific names already in use. Work of different authors including Migula (1900); Chester (1901); Winslow and Rogers (1905–1906); Winslow and Winslow (1908); Kligler (1913); the report on the Committee on Classification (1917); Buchanan (1917); Winslow, Rothberg and Parsons (1920); Hucker (1924), and Bergey (1925) were used as guides in selecting specific names for my strains.

In the orange group there were 9 strains that were gelatin and

lactose positive; all of them produced sufficient acid to curdle milk, and most of them (7) also reduced nitrates. The name *Staphylococcus aureus* was applied to them, but to find a proper name for the strains in this group that were gelatin-negative, but lactose-positive is difficult. Winslow, Rothberg and Parsons (1920) found only a few strains with such characteristics and suggested leaving them nameless at that time. They, like most investigators, applied the name *Staphylococcus aurantiacus* to an orange strain that was gelatin and lactose negative. However, Hucker (1924) in his work finds that *M. aurantiacus* is closely related to *M. aureus*, but differs in its ability to liquefy gelatin, and also differs serologically. Although serological tests were not employed in this work, the name *M. aurantiacus* was applied to 4 strains that were gelatin-negative, but lactose-positive; also to one strain that was gelatin- and lactose-negative. Of these 5 strains 2 only reduced nitrates. The only strain of *Staphylococcus citreus* that I have in this series liquefied gelatin; reduced nitrates, and produced sufficient acid to curdle milk.

In the white group there were 3 strains of *Staphylococcus albus*, all of which fermented lactose; liquefied gelatin; reduced nitrates, and produced sufficient acid to curdle milk. There were 2 more strains with the same characteristics, except that both of them peptonized milk rather rapidly. The name *M. caseolyticus*, proposed by Evans (1916), was applied to these two strains. Three strains were identified as *M. ureae*; all of them decomposed urea, produced sufficient acid to curdle milk, but failed to reduce nitrates, or liquefy gelatin. There was one strain that liquefied gelatin and decomposed urea, but did not reduce nitrates, nor did it ferment lactose or mannitol; litmus milk was unchanged. The name *M. ureae liquefaciens* (Flügge) was applied to it. Three strains were classified as *Staphylococcus epidermidis* (Kligler). They did not liquefy gelatin, but reduced nitrates; all of them fermented lactose, mannitol, maltose and glycerol; two of them fermented salicin. Two strains were classified as *M. candidus*. They did not liquefy gelatin, nor did they reduce nitrates. One strain was classified as *M. tetragenus* on account of its typical cell-arrangement.

These 30 strains were isolated in connection with the following infections: prostatitis; urethritis; pyelonephritis; urinary calculus; ulcerated cystitis; epididymitis and prostatic abscesses. It was found that, in most cases, orange strains produced more severe infections than white, although certain strains in the white group are among the most virulent in the whole series.

SUMMARY AND CONCLUSIONS

Thirty strains of Gram-positive cocci isolated from thirty cases of genito-urinary infection were diagnostically studied. Following are the principal results:

1. The most prevalent type of infection in this series is prostatitis. However, no correlation is yet established between this type of infection and the strain of cocci isolated in connection with it.

2. Strains in this series have a tendency to ferment carbohydrates strongly. The following carbohydrates were fermented by almost all the strains: glucose, sucrose, maltose, mannitol and glycerol.

3. The ability of strains in this series to decompose urea was very pronounced.

4. On the whole, the white strains exhibited more variation in their cultural characteristics than the orange.

5. The orange group proved to have a higher percentage of gelatin-liquefying; nitrate-reducing; milk-coagulating, and urea-decomposing strains. In general, the orange strains were more active, and the infections, in connection with which they were isolated, were more severe.

REFERENCES

BAYNE-JONES, S., AND ZINNINGER, PAULINE 1921 Bull. Johns Hopkins Hosp., **32**, 299.

BERGEY, D. H. 1925 Manual of Determinative Bacteriology. Baltimore.

BROWN, J. HOWARD 1919 Monograph 9, Rockefeller Inst. Med. Research.

BUCHANAN, R. E. 1916 Jour. Bacteriol., **2**, 603.

CHESTER, F. D. 1901 Manual of Determinative Bacteriology, New York.

Committee on Classification. 1917 Jour. Bacteriol., **2**, 505.

DUDGEON, L. S. 1908 Jour. Pathol. and Bacteriol., **12**, 242.

EVANS, ALICE 1916 Jour. Infec. Dis., **18**, 437.

FLÜGGE, C. 1896 Die Mikroörganismen, **2**, 173.
HUCKER, G. J. 1924 Studies on the Coccaceae: I–IV, Geneva, N. Y. State Agri. Exper. Station Tech. Bull., June.
JULIANELLE, L. A. 1922 Jour. Infec. Dis., **31**, 256.
KLIGLER, I. J. 1913 Jour. Infec. Dis., **13**, 432.
KRUSE, W. 1896 Die Mikroörganismen, **2**, 475.
KUTSCHER AND KONRICH 1904 Ztschr. f. Hyg. u. Infektionskr., **48**, 249.
Manual of Pure Culture Study 1924 Geneva, N. Y., B-10; 11; B-23–25.
MELLON, R. R., AND CALDWELL, D. 1926 Jour. Bacteriol., **12**, 409.
MIGULA, W. 1900 System der Bakterien. Jena, Bd. 2.
MIQUEL, P. 1888 Études sur la fermentation ammoniacale et sur les ferments l'urée. Ann. de Micrographie, **1**, 415.
NEISSER, M., AND WECHSBERG, F. 1901 Ztschr. f. Hyg. u. Infektionskr., **36**, 299.
PASTEUR, L. 1860 Compt. rend. de l'Acad. Science, **50**, 849.
PASTEUR, L. 1862 Ann. de Chim. et Phys., **64**, 52.
WINSLOW, C.-E. A., AND ROGERS, ANNE 1905 A Revision of the Coccaceae. Science, N.S. **21**, 669.
WINSLOW, C.-E. A., AND ROGERS, ANNE 1906 Jour. Infec. Dis., **3**, 485.
WINSLOW, C.-E. A., ROTHBERG, W., AND PARSONS, ELIZABETH 1920 Jour. Bacteriol., **5**, 145.
WINSLOW, C.-E. A., AND ROGERS, ANNE 1908 The Systematic Relationships of the Coccaceae. New York.

THE EFFECT OF FILTRATES OF CERTAIN INTESTINAL MICROBES UPON BACTERIAL GROWTH

MARGARET F. UPTON

Department of Bacteriology and Public Health Washington University School of Medicine

Received for publication, October 20, 1928

INTRODUCTION

In undertaking an investigation of the intestinal flora of normal breast-fed and artificially fed infants (work about to be published), the writer was impressed with the apparently great inhibitory action which *Bacillus bifidus* exerts upon the development of other intestinal bacteria in laboratory cultures. If one prepares two inoculated lactose-liver-agar plates, using the spiral streak method (Varney, 1927), from undiluted intestinal material from a healthy breast-fed infant a week or more old, and incubates one plate aerobically and the other anaerobically, the resulting cultures are strikingly different. As has been pointed out by Tissier and Dreyfus (1925) and by other authors, in the intestinal flora of such infants only three types of bacteria are constantly present: *Bacillus bifidus*, *Bacillus coli*, and the enterococcus or *Micrococcus ovalis*. As *B. bifidus* is an anaerobe, no colonies of this type develop upon the aerobic plate. Consequently this culture shows a relatively even distribution of *B. coli* and *M. ovalis*. On the anaerobic plate, however, enormous numbers of small colonies of *B. bifidus* appear and only occasional colonies of *B. coli* and *M. ovalis*. The proportions of the different colonies are closely comparable to those of bacteria observed on the Gram-stained direct smear from fecal material where one encounters *B. bifidus* in almost pure state. As there are doubtless approximately the same number of *B. coli* and *M. ovalis* organisms in the two bits of material streaked, there is evidently an inhibition of growth due to the presence of *B. bifidus* when conditions favor-

able to its development are present. *B. coli* and *M. ovalis* grow anaerobically as well as under aerobic conditions. Similar plates made from feces from a bottle-fed child differ very little from each other with respect to the development of these two bacteria. The anaerobic plate shows *B. bifidus* but usually it occurs in such small numbers that its presence has no appreciable effect upon the other types.

This inhibitory effect of *B. bifidus* upon other bacteria was investigated by Kling (1914). He concluded from his work that the bactericidal action exerted was due entirely to the acid-producing properties of the bacterium, since, when cultures were neutralized, no inhibition of other forms took place. He made no distinction between "acid-producing properties" in terms of hydrogen ions and the molecular character of the acids involved. As was found in experiments about to be described, neutralizing the bacterial culture (or filtrate) will indeed destroy the bactericidal property but it appears that when the hydrogen-ion concentration is only slightly lowered with alkali, factors other than the pH become apparent which tend to discourage bacterial growth.

EXPERIMENTAL

The three organisms used in this study were *B. coli* (fermenting sucrose), *M. ovalis* and *B. bifidus*. They were freshly isolated strains from the stools of normal infants. The same strains were used throughout, except that in the work with the organic acids, and in one experiment with the filtrates, different strains of *B. coli* and *M. ovalis* were used for inoculating purposes.

The medium for these experiments was selected with special reference to the requirements of *B. bifidus*, as that organism is the most difficult of the three to cultivate. As a result of some experimenting it was found that after isolation on lactose-liver agar the organism could be transferred to lactose-peptone broth. A broth containing Difco proteose peptone (0.5 per cent) and dipotassium phosphate (0.5 per cent) was adjusted to pH 7.0 with sulfuric acid. A 20-per cent solution of lactose in distilled water was sterilized by passage through a Berkefeld filter to avoid

any possible hydrolysis by heating. The lactose was then added to the sterile broth so that there was a final concentration of 1 per cent. This lactose medium was used in all the experiments described in this paper. Each of the three organisms was inoculated into flasks. *B. coli* and *M. ovalis* were grown aerobically at 37°C. for from three to five days. It was found that the pH of the cultures remained the same after three days' incubation. *B. bifidus* was incubated in an anaerobic phosphorous jar for 4 days or until growth appeared heavy. At the end of the incubation period only *B. coli* appeared to be still viable. The broth containing the organisms was then centrifuged and passed through a sterile Berkefeld filter. The pH of the *B. coli* filtrate was 5.0 to 5.3, of the *M. ovalis*, 4.8 to 4.9, and of the *B. bifidus*, about 4.0. After some preliminary work it was decided to run experiments at three representative hydrogen-ion concentrations. Accordingly, flasks of each filtrate were adjusted by sodium hydroxide or hydrochloric acid to pH 4.5 pH 5.0 and pH 6.0. Gillespie's standards of pH values, using methyl red as indicator (Clark, 1922), were employed in estimating pH. The pH value 4.5 was selected because with this acidity, *B. coli* is still able to develop, although inhibited somewhat by the high hydrogen-ion concentration. The "critical zone" for *B. coli* is described by Cohen and Clark (1919) as lying between pH 4.6 and 5.0 in media adjusted with hydrochloric acid. The pH value 5.0 is approximately the hydrogen-ion concentration of the breast-fed infant's feces and pH 6.0, that of the artificially fed infant's. In fact, in experiments where results were estimated at pH 7.0, there was practically no difference between pH 6.0 and pH 7.0 so that neutralizing effects are apparent at the lower pH value. Control flasks, using uninoculated broth, were adjusted in the same way. Four sets of sterile test tubes, containing 5 cc. each of filtrate at each pH, were set up. Three sets were inoculated with equal amounts (usually 0.2 cc.), each, of a suspension of one of the three organisms from a slant washed down with broth. The fourth set was left uninoculated as a control. At first the sets containing *B. coli* and *M. ovalis* were incubated aerobically, but it was found that the organisms remained viable longer under

anaerobic conditions and there was no loss in volume by evaporation. Consequently the sets were incubated in the anaerobic jar.

After twenty-four hours the turbidity in each tube was recorded, as indicated in table 1. It is quite evident that *B. coli* and *M. ovalis* do not produce the same degree of turbidity. The expression "++++" therefore indicates a maximum turbidity for the organism under consideration and the other symbols refer to the turbidity, relative to this maximum. Every day, beginning with the first after inoculation, one loopful of culture from each tube was streaked on a portion of lactose agar plate. This transplanting was done until no colonies grew from the tubes. These transplants gave a good idea of the viability of the

TABLE 1

Growth of B. coli and M. ovalis in filtrates and broth as shown by turbidity in twenty-four hours

MEDIUM	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
B. coli filtrate	Clear	±	++++	Clear	Clear	++++
B. bifidus filtrate	Clear	±	+++	Clear	Clear	+++
M. ovalis filtrate	Clear	+	+++	Clear	Clear	++
Lactose broth	++	++	++++	±	±	++++

organisms. Organisms may remain alive for many days in a culture which shows practically no turbidity. One often sees experiments recorded in the literature where the clarity of a tube is taken as an indication of sterility, whereas, often, the organism may still be viable without visible growth. The amount of growth gave a good indication of the relative numbers of living bacteria in the tubes and an approaching dying-out of the culture could be predicted by a falling-off in the number of colonies.

Similar tests, using *B. bifidus*, were attempted but owing to the peculiarities of this organism difficulties were encountered which made the meager results obtained unreliable for comparative purposes. Usually, the organism failed to grow, or, if growth took place, it died out so quickly in the tubes that transplants for

purposes of testing viability were unsatisfactory. Probably the small volume of culture used tended to restrict growth and what growth took place was not sufficient to be detected by the rather rough methods employed.

Table 1 shows, by the turbidity produced in twenty-four hours, the effect of the filtrates upon growth of the bacteria. As may be seen by comparing the filtrates at pH 4.5 and 5.0 with the corresponding tubes of lactose broth, bacterial growth is definitely inhibited in the filtrates, whereas in broth it is allowed to proceed. At pH 6.0, or, as the reaction approaches neutrality, growth in the filtrates is little less marked than in the broth. Table 2 corroborates these results by showing that bacteria remain alive longer in broth than in the filtrates at pH 4.5. It, moreover, indicates

TABLE 2

Viability of B. coli and M. ovalis in filtrates and broth as shown by number of days the organisms remained alive

MEDIUM	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
B. coli filtrate	0	1	9	0	5	9
B. bifidus filtrate	0	1	5	0	9	9
M. ovalis filtrate	1	2	6	2	9	9
Lactose broth	4	5	4	7	5	5

that, as the filtrates approach neutrality, they may even have a more beneficial effect upon the bacteria than broth alone. *M. ovalis* is less inhibited than *B. coli* at pH 5.0, as shown in table 2. The fact that the organism remains viable for a long time at this pH whereas the broth shows little evidence of cell multiplication, indicates that the effect is inhibitive rather than bactericidal.

This brings us to the question of what metabolic products yielded by the bacteria may be the means of bringing about such results, for it is quite evident that the hydrogen-ion concentration alone cannot in these experiments account for the inhibition. In the presence of fairly large amounts of carbohydrates, it has been long known that bacteria produce but slight, if any, nitrogenous changes in the substrate. The buffer used, only 0.5

per cent, would not be sufficient to allow even the nitrogenous changes described by Slanetz and Rettger (1928). The carbohydrate was not used up in these cultures, as was proved by a positive Benedict's sugar test on the filtrates at the end of the incubation period. Moreover the aciduric organisms, *M. ovalis* and *B. bifidus*, have been shown by Kendall (1921) to have practically no ability to alter nitrogenous substances. The acid-producing properties, of the three organisms studied are, however, well known and it was decided to determine roughly the proportions of volatile and lactic acids produced from lactose sugar by these organisms.

METHOD FOR DETERMINING VOLATILE ACIDS

The method of precipitating the proteins was a modification of a method for determining the volatile acids present in adult feces developed by W. H. Olmsted in work to be published. To 250 cc. of a five- or six-day culture were added 150 cc. of 10 per cent mercuric chloride (in 4 per cent HCl). A thick suspension of calcium hydroxide was added until the solution was decidedly alkaline, and the volume was made up to 500 cc. After centrifuging, 200 cc. of the supernatant fluid, made acid with sulfuric acid, were treated with hydrogen sulfide, aerated, filtered and diluted to 250 cc.

To 100 cc. of filtrate were added 70 grams magnesium sulfate and 2 cc. of 50 per cent sulfuric acid. The mixture was steam-distilled at the rate of 100 cc. in nine to ten minutes. The 100-cc. fractions were titrated with tenth-normal and one-hundredth-normal sodium hydroxide. A filtrate from a blank of uninoculated broth was prepared and distilled in the same way, and the readings deducted from the readings on the cultures for the final result.

METHOD FOR DETERMINING LACTIC ACID

Mercuric nitrate solution was used in precipitating the protein. To 50 cc. of broth or culture were added 5 cc. of mercuric nitrate solution (in 1 liter, 160 cc. of concentrated HNO_3 220 grams HgO, 60 cc. of 5 per cent NaOH); 30 per cent sodium hydroxide was

added until a yellow precipitate appeared but not enough to cause the solution to become alkaline. After making up to 100 cc., barium carbonate was added until the solution was neutral to litmus. After filtering, sodium sulfate was added to precipitate the barium sulfate. The solution was then made acid with concentrated sulfuric acid and treated with hydrogen sulfide to remove the mercury; the solution was then aerated, a drop of 10 per cent copper sulfate solution added and the solution filtered. To each 10 cc. of filtrate, was added 1 cc. of 20 per cent copper sulfate and enough calcium hydroxide to make it alkaline. This removed the sugar. Five cubic centimeters of the filtrate were

TABLE 3

Volatile and lactic acids recovered from six-day anaerobic cultures in 1 per cent lactose-buffered-peptone broth, expressed in terms of 0.1 N NaOH per 100 cc. culture

ORGANISM	TITRATED ACIDITY	VOLATILE ACIDS		LACTIC ACID		PER CENT OF TITRATED ACIDS RECOVERED
		Total	Per cent of titrated acids	Total	Per cent of titrated acids	
B. coli	26.5	12.4	46.8	2.9	10.9	57.7
M. ovalis	25.3	3.7	14.6	16.7	66.2	80.8
B. bifidus	100.2	64.6	64.6	22.6	22.6	87.2

used for the lactic acid determination. The method and apparatus used was that described by Friedemann, Cotonio and Shaffer (1927). A blank was run on a filtrate from uninoculated broth and the readings deducted. One-hundredth-normal iodine was used for titrating.

The results obtained with a representative strain of each type are noted in table 3. While actual figures varied somewhat on the several different cultures, the results are consistent. One can readily see that the three organisms differ markedly with regard to the utilization of the carbohydrate. *B. bifidus* produces the largest titratable acidity, *B. coli* and *M. ovalis* about equal amounts. *M. ovalis* produces less lactic acid than does *B. bifidus*, but this acid forms a greater percentage of the total acid production. *B. bifidus* produces large amounts of volatile

acids both in actual amount and in proportion to the titrated acidity. *B. coli* produces more volatile acids than *M. ovalis* but much less lactic acid. Comparing the distillation curves of the volatile acids produced by *B. bifidus* with the curves of the volatile acids as determined by W. H. Olmsted (in work to be published) we may conclude that the distillate probably consists largely of formic and acetic acids while that of *B. coli* appears

TABLE 4

Growth of B. coli and M. ovalis in adjusted acid broths (0.02 N) as shown by turbidity in twenty-four hours

ACID	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
Formic	Clear	Clear	++++	Clear	+	++++
Acetic	Clear	Clear	+++	Clear	+	++++
Lactic	Clear	++	+++	Clear	+++	++++
HCl	++	+++	+++	±	++	++++

TABLE 5

Viability of B. coli and M. ovalis in adjusted acid broths (0.02 N) as shown by number of days the organisms remained alive

ACID	B. COLI			M. OVALIS		
	pH 4.5	pH 5.0	pH 6.0	pH 4.5	pH 5.0	pH 6.0
Formic	2	7	7	2	4	4
Acetic	1	5	7	7*	5	5
Lactic	2	7	5	5	4	4
HCl	6	3	3	7	7	6

* Only a very few colonies were obtained on transplants after third day.

to be largely acetic. It is to be expected that little formic acid would be recovered from the *B. coli* culture as it has been shown that the formiase present breaks this substance down into carbon dioxide and hydrogen. The amount of volatile acids distilled from the culture of *M. ovalis* was so small that no opinion could be drawn as to their character, except that they are probably of the lower fatty-acid series.

Apparently, then, filtrates from these organisms contain con-

siderable quantities of organic acids and it seemed advisable to determine if media containing these acids would have any particular effect upon bacteria. Accordingly the following experiment was set up.

Normal solutions of acetic, formic, lactic and hydrochloric acids were prepared and added to 1 per cent lactose-peptone buffered-broth so that a final concentration of two-hundredth-normal was obtained for each acid. A quantity of each solution was then adjusted to pH 4.5, 5.0, and 6.0 with NaOH. An experiment similar to that with the filtrates, but using the above solutions instead of the filtrates, was then set up. The tubes containing HCl acted as controls on the organic acids. The tubes were inoculated and incubated as before. Tables 4 and 5 give the results expressed in the same manner. It may be readily seen that there is a definite inhibition of growth at pH 4.5, especially of *B. coli*, in the cultures containing formic and acetic acid, verified as before by length of life as determined by transplants. The hydrochloric-acid broth adjusted to this pH permits the bacteria to grow. Lactic acid, however, seems to have less inhibitory action than acetic and formic acids. However, as the reaction becomes more nearly neutral, the presence of the organic anion apparently makes conditions more favorable than in the tubes containing the inorganic acid. Possibly, too much emphasis should not be placed upon this phenomenon as expressed by the number of days for which the strains were viable. However, it was noted repeatedly in similar experiments. It is possible that the salts formed as these acids are neutralized have a buffering effect, but in the concentration occurring in these experiments such an effect could be but slight. The pH of these tubes was observed at the end of the experiments. At the same initial pH the final pH of the four acids varied somewhat in different experiments. With *B. coli* at pH 6.0, the organic-acid tubes in one experiment were slightly more alkaline than the HCl tube, but the difference was slight and possibly could be accounted for by errors in colorimetric reading and by the variation in abundance of growth in the tubes.

DISCUSSION

The presence of certain organic acids in culture media has much the same effect upon the growth of the intestinal bacteria studied at pH 4.5 and 5.0 as do filtrates from cultures in which these lactose-fermenting bacteria themselves have been growing. Bacterial growth is but slightly inhibited in lactose broth containing the same concentration of hydrochloric acid at these pH values. The effect of these organic acids upon the development of *B. coli* and *M. ovalis* may have some bearing upon the prevalence of these bacteria in the intestinal tract as illustrated by a comparison of their relative numbers in the feces of the artificially and breast-fed infants. The breast-fed baby has an overwhelming predominance of *B. bifidus* and in cultures prepared from fecal material under anaerobic conditions few colonies of *B. coli* and *M. ovalis* are found. If the conditions of growth in the intestine are at all similar to those in lactose-buffered broth it seems reasonable to assume that large amounts of the lower volatile acids are produced. In fact, Bosworth and his coworkers (1922) encountered formic and acetic acids in analyses of infants' stools. They endeavored to find the source of the formic acid in these stools and looked upon *B. bifidus* as a factor in its production. Their experiment, however, was based on identification of the volatile acids produced by this organism from citric acid and was disappointing in the lack of formic acid found. As has been pointed out by Kendall (1921), lactose is continually present in the intestinal tract of the normal nursling. A substitution of some other sugars invariably leads to a replacement by other organisms. No doubt, then, the formic acid found to be present in the infant's intestine is derived largely by *B. bifidus* from lactose fermentation. The fact that *B. bifidus* is practically a carbohydrate-obligate substantiates this idea. The concentration of these acids used in the experiments with the acids alone was much less than that actually produced by *B. bifidus* in the cultures. The pH of the normal breast-fed infant stool is about 5.2. It is possible that higher up in the intestinal tract the reaction is more acid. Under conditions of such acidity, according

to the above experiments, the inhibitory effect of the volatile acids would be present. Apparently *B. bifidus* in the intestine is favored by the presence of these organic acids at a high hydrogen-ion concentration and doubtless other unknown conditions, so far not reproduced in the test tube, may have a beneficial effect. Cruickshank (1925) mentions that glucose broth acidified with acetic acid inhibited *B. coli*, but allowed *B. bifidus* to develop.

On the other hand, in the artificially fed infant, one finds the predominating organism in most cases to be *M. ovalis*, with *B. coli* almost as prevalent. *B. bifidus* is usually present but to no very constant or marked degree. The pH of the feces is 6 to 8. In such cases the organic acids produced during fermentation tend to become neutralized and so fail to inhibit the intestinal forms more common in the adult intestine. It seems that organic anions tend to be inhibitory under acid conditions, but lose this power when more of the salts are present as neutrality is approached. Bial, as cited by Winslow and Lochridge (1906) noted a diminution of antiseptic action of acids when neutral salts were added. These writers considered the toxic action of organic acids due to the anion or undissociated molecule. Cohen and Clark (1919) felt that the free acetate radicle may exert a synergic effect on the disinfecting power of the hydrogen ion.

Perhaps not too much significance should be placed on the following fact; yet it is interesting to note that the filtrate from *M. ovalis* had less toxic effect than that of the other two organisms. According to the analyses of the experiments, *M. ovalis* produces the largest percentage of titratable acids, as lactic acid rather than volatile. As was mentioned before, lactic acid has less effect upon the development of bacteria than the other organic acids tested.

SUMMARY

1. *B. coli* and *M. ovalis* are inhibited in bacterial filtrates of cultures of *B. coli*, *M. ovalis* and *B. bifidus* at a pH value of 4.5 and 5.0.

2. The inhibition appears to be due to some factor in addition to that caused by an increase in H-ions.

3. Acetic and formic acids show an effect similar to that shown by filtrates, which disappears as the acids tend to become neutralized.

4. Lactic acid has less inhibitory effect than the other acids studied.

5. Under the same conditions, *B. coli*, *M. ovalis* and *B. bifidus* utilize lactose differently, giving different proportions of volatile and lactic acids.

6. The question is discussed as to the connection between the presence of large amounts of volatile acids presumably produced by *B. bifidus* in the intestine of the breast-fed infant and the inhibition of *M. ovalis* and *B. coli* in that locality. The prevalence of these last two organisms in the feces of the artificially fed infant, where the reaction is less acid, would seem to correlate with the results obtained from experiments outlined in this paper.

The writer is greatly indebted to Dr. William H. Olmsted, Dr. Ethel Ronzoni, and Dr. Edward S. West for valuable advice and assistance in regard to the chemical procedures described in this paper.

REFERENCES

BENEDICT, S. R., AND OSTERBERG, E. 1918 A method for the determination of sugar in normal urine. Jour. Biol. Chem., **34**, 198.

BOSWORTH, A. W., WILDER, H. K., (ET AL.) 1922 Bacterio-chemical study of acid stools excreted by breast-fed and bottle-fed infants. Amer. Jour. Dis. Child., **23**, 323.

CLARK, W. M. 1922 Determination of Hydrogen Ions. Baltimore.

COHEN, B., AND CLARK, W. M. 1919 Growth of certain bacteria in media of different hydrogen ion concentration. Jour. Bacteriol., **4**, 409.

CRUICKSHANK, R. 1925–1926 *Bacillus bifidus:* its characters and isolation from the intestine of infants. Jour. Hyg., **24**, 241.

FRIEDEMANN, T. E., COTONIO, M., AND SHAFFER, P. A. 1927 The determination of lactic acid. Jour. Biol. Chem., **73**, 335.

KENDALL, A. I. 1921 Bacteriology—General, Pathological and Intestinal. Philadelphia.

KLING, A. 1914 Du rôle physiologique joué par le *B. bifidus* dans le canal intestinal. Ann. de l'Inst. Pasteur, **28**, 797.

SLANETZ, C. A., AND RETTGER, L. F. 1928 Bacterial metabolism—The influence of phosphate buffer in carbohydrate-free and in glucose-containing media. Jour. Bacteriol., **15**, 297.

TISSIER, H., AND DREYFUS, S. 1925 Sur les streptococques de la flore intestinale des nourissons au sein. Compt. Rend. Soc. de biol., **92**, 476.

VARNEY, P. L. 1927 A new spiral streak plate method of isolating bacteria by means of an inoculating machine. Jour. Infect. Dis., **41**, 190.

WINSLOW, C.-E. A., AND LOCHRIDGE, E. E. 1906 The toxic effect of certain acids upon typhoid and colon bacilli in relation to the degree of their dissociation. Jour. Infect. Dis., **3**, 547.

THE USE OF CULTURE MEDIA MADE FROM COMMERCIAL DRIED YEAST AS A ROUTINE SUBSTITUTE FOR MEAT INFUSION PEPTONE MEDIA

JAMES M. NEILL, JOHN Y. SUGG, LURLINE V. RICHARDSON, AND WILLIAM L. FLEMING

From the Department of Bacteriology and Immunology of Vanderbilt University Medical School, Nashville

Received for publication, November 14, 1928

During the past few years many batches of meat infusion peptone broth prepared in our laboratory have failed to grow pneumococci and other kinds of bacteria that usually flourish in good batches of this medium. Because of this experience, media prepared from commercial dried yeast have been tested. Throughout a year's use they have proved to be uniformly reliable and efficient substitutes in all routine bacteriological procedures for which meat infusion peptone media are commonly used. The purpose of this paper is to report the method of preparation and the advantages and limitations of these yeast media.

METHOD OF PREPARATION

A number of different procedures have been tried, but the following methods were chosen as the simplest and most rapid.

I. Preparation of "five per cent yeast" broth

The medium found satisfactory as a routine substitute for meat-infusion-peptone broth was prepared by the following steps. For convenience, the amounts of material are expressed in terms of 10 liter lots of medium.

1. Infusion of the dried yeast. Warm 10 liters dist. H_2O to 40°C.; add 500 grams Fleischmann's dried yeast[1] to the H_2O and infuse at

[1] The dried yeast was obtained from the Fleischmann Yeast Company. It contains no starch and consists of dried yeast cells and is the same material as that distributed commercially for feeding cattle and other stock. Four different lots

40°C. for thirty minutes; raise temperature to 50°C. and continue the infusion for ninety minutes.

2. Preliminary adjustment of pH of the infusion before boiling. Add 8 per cent (2.0 *N*) NaOH to the infusion until phenol red gives a faint pink when added to samples of the turbid yeast suspension (about 40 cc. 2.0 *N* NaOH are usually required). Add 30 grams $Na_2HPO_4 \cdot 12$ H_2O previously dissolved in 100 to 200 cc. of warm H_2O.

3. Boiling and preliminary filtration of yeast infusion. Raise temperature to boiling; stirring is necessary when the temperature approaches boiling point. Boil for one to three minutes. Filter through paper.

4. Adjustment of volume. Add H_2O to compensate for loss due to evaporation and filtration, until volume is 9.5 liters.

5. Final adjustment of pH. The broth is perfectly clear after the filtration in step 3, and colorimetric tests always show the pH to have dropped below 7.0. Adjust by addition of NaOH until pH is from 7.2 to 7.4. Add 150 grams $Na_2HPO_4 \cdot 12$ H_2O previously dissolved in 500 cc. warm H_2O. The addition of this phosphate usually raises the pH to about 8.0 with media prepared from most lots of the dried yeast. If a final pH of 7.8 is desired, the broth should be readjusted by addition of alkali, or acid if it is not pH 8.0 after addition of the phosphate at this stage. (We prefer to adjust the pH with NaOH added in the order described to a point sufficient to enable the last addition of phosphate to raise the pH to 8.0. This point (usually pH 7.2 to 7.4) varies to some extent with different lots of yeast, but is fairly constant in different batches prepared from the same lot of dried yeast.)

6. Precipitation at 120°C. before final filtration. Distribute the adjusted broth into large flasks and autoclave five minutes at 120°C.

---

have yielded uniformly good culture media, so that it is probable that all similar material will give good results. There was some variation in the buffer curves and in the content of fermentable substance in media prepared from different lots of the dried yeast, but none of these variations introduced any significant complications. Apparently, the yeast should be obtained as a reasonably fresh preparation; although lots of dried yeast that had been in the laboratory for over six months proved satisfactory, one lot that had been stored for about three years in a warm room furnished inferior media.

The dried yeast from which most of the media has been prepared, was in the form of coarse granules. Broth made from one lot of finely powdered yeast seemed equally good from the standpoint of its nutrient qualities, but the media made from the powdered material had the disadvantage of slower filtration than that made from the granular material.

This causes precipitation of material that would otherwise be precipitated during the final sterilization. The precipitate is well separated from the clear supernatant and the medium filters as rapidly as ordinary broth. This heating usually causes a drop in pH from 8.0 to 7.9.

7. Final sterilization. Distribute the filtered broth in the desired containers and sterilize twelve to fifteen minutes at 120°C. This usually causes the pH to drop to 7.8.

II. Preparation of agar medium with "five per cent yeast" broth as base

1. Preliminary softening of agar. Add 250 grams agar to about 2 liters H_2O and let it soak until time for its addition to the medium.

2. Preparation of yeast broth base. Proceed as in steps 1, 2, and 3 described under I. (Use 10 liters H_2O as the decrease in volume will compensate for the H_2O added to the agar.)

3. Adjust volume of broth, if decrease in volume after filtration has reduced it lower than 7.5 liters.

4. Adjustment of pH. Raise pH to about 7.4 to 7.5. Add 150 grams phosphate dissolved in about 500 cc. H_2O as in step 5 in I. This should bring reaction to at least pH 8.1, and if final pH of 7.8 is desired, the reaction should be readjusted if it is now below pH 8.0.

5. Addition of agar. Add agar softened by previous soaking in step 1.

6. Solution of agar and precipitation before final sterilization. Autoclave for fifteen minutes at 120°C. instead of the 5 minutes employed in step 6 of I, in order to dissolve the agar. Filter through cotton and test pH which should now be pH 7.8 to 7.9.

7. Final sterilization. As in analogous step in I.

III. Preparation of "twelve per cent yeast" broth

This medium is prepared by essentially the same procedure as described in I for "5 per cent yeast" broth with the exception that the amount of dried yeast is increased to 120 grams per liter. The additional amount of yeast makes the preparation of this medium much more time-consuming, for filtration which is rapid with the "5 per cent" medium is slow with the "12 per cent" medium.

IV. Preparation of agar medium with "twelve per cent yeast" broth as base

Agar medium may be prepared by the addition of 2.5 per cent agar to the "12 per cent yeast" broth.

The uniform reliability of every batch of "5 per cent yeast" broth is sufficient justification for its substitution for meat infu-

sion peptone media for routine use in laboratories which have difficulty in the preparation of uniformly satisfactory media from meat. However, in addition to the advantage of reliability, the yeast broth possesses the advantage of cheapness. The total cost of the materials for one liter is 9 cents (7.6 cents for the yeast and 1.4 cents for the phosphate), or approximately the same as that of the 10 grams of peptone contained in one liter of meat infusion peptone media. Hence, by the use of yeast broth in place of meat infusion peptone broth, one saves the entire cost of the meat which varies from 15 to 30 cents per liter. The "12 per cent" media are described because for some special purposes they possess the advantage of a somewhat more rapid and luxuriant bacterial growth. In many instances, the richer medium is a disadvantage and in general practice, the advantage of the "12 per cent" over the "5 per cent" medium is not sufficient to justify the greater labor and expense involved in its preparation.

FERMENTABLE CARBOHYDRATE, BUFFER VALUE, AND FINAL PH OF CULTURES GROWN IN THE DESCRIBED YEAST MEDIA

The amount of reducing sugar in "5 per cent yeast" broth is insignificant as it is not detectable in tests of 0.5 cc. of the broth layered on 2.0 cc. of Benedict's solution; slight traces are present in some batches of the "12 per cent" broth. The amount of reducing sugar, is never sufficient to account for the growth-promoting properties of the yeast broth. Tests for glycogen proved positive in all lots of yeast broth. The presence of fermentable carbohydrate has been recognized by other workers in the preparation of different yeast media and some of them have employed preliminary autolysis of the yeast as a means of removing the fermentable substance. Our main purpose, however, was to obtain a medium that was simple in preparation and preliminary autolysis of the yeast seemed to complicate the method to too great an extent.

The amount of phosphate in the medium is sufficient to compensate for the production of a considerable amount of acid, and in the "5 per cent" broth none of the common bacteria reach a

final pH sufficiently acid to interfere with most routine bacteriological procedures. (The production of acid varies to some extent with the lot of dried yeast from which the medium is prepared, but the following pH values represent that obtained in most of the batches of "5 per cent yeast" broth that we have made: Pneumococci, pH 6.6 to 7.0; scarlet fever and other hemolytic streptococci, 6.3 to 6.7; staphylococci, 6.7 to 7.0; diphtheria bacilli, 7.2 to 7.6; anthrax bacilli, 6.6 to 6.9; Friedländer, colon bacilli, and paratyphoid, 6.3 to 6.7; typhoid, 6.6 to 6.9; Flexner dysentery, 6.3 to 6.4; Shiga dysentery 6.7 to 6.8; tetanus, 6.8 to 7.2; Welch bacillus, 6.5 to 6.8; vibrion septique, 6.3 to 6.6.) Hence one can simply accept the production of some acid as a property of the medium that does not interfere with its routine use. Rather than to complicate the method of preparation by attempts to remove the source of acid, it seemed preferable to use another culture medium for any purpose in which the acid production was a real disadvantage. The amount of acid produced in the "12 per cent yeast" broth, however, is significant; with some bacteria, the pH drops to below 6.0; pneumococci, for example, usually giving a final pH from 5.6 to 6.0. For some purposes, however, even with pneumococci, this disadvantage can be circumvented by using the cultures at an early growth stage before the final high acidity is reached.

TESTS OF POSSIBLE SEROLOGICAL COMPLICATIONS

Since Fleischmann's dried yeast was the source of Mueller's preparation of the specific yeast carbohydrate (Mueller and Tomcski, 1924), some of the same serologically reactive substance must be contained in the described yeast broth. This suggested two possible sources of danger. First, that immunization with yeast broth cultures of bacteria might give rise to anti-yeast precipitins reactive with the broth itself. Second, that the reactive yeast carbohydrate while derived from yeast, might be chemically related to the specific *S* substance of certain bacteria and consequently cause some serological reaction between yeast broth and the antibacterial serum.

We have investigated both these possibilities. The first appears to be unimportant as a source of error. Six rabbits have been immunized with the described yeast broth; two of them with 4 courses including 24 injections of 10 cc. of the broth. None of the animals developed any detectable traces of precipitins against the yeast broth. Apparently, the dissociated *S* substance which Mueller found to be devoid of antibody-invoking properties represents the only yeast antigen (haptene) present in significant amounts in the broth. If use of the yeast broth is limited to routine purposes, the possibility of the presence of traces of other yeast antigens is not important.

The second possibility would be encountered in the agglutination of cultures grown in yeast broth against an antibacterial serum that might happen to include precipitins reactive with the *S* substance contained in yeast broth. That this is a real danger is due to two facts: (1) that Fleischmann's yeast possesses a certain serological relationship to Type II pneumococci; (2) that the serum from many normal rabbits possesses agglutinins for cultures of the *yeast cells* grown in any kind of broth. (These inter-reactions have been reported by Sugg and Neill (1929)). However, while some normal rabbit sera and Type II anti-pneumococcus horse serum do agglutinate *yeast cells*, none that we have tested precipitate the described "5 per cent yeast" broth. The yeast broth is precipitated by antiserum prepared by immunization with yeast cells (as would be expected from Mueller's work with yeast and from Avery and Heidelberger's work with pneumococci), but not by any of over 100 antibacterial antisera that we have tested. Hence, unless one attempted to agglutinate bacterial cultures grown in yeast broth against an anti-yeast-cell serum, the use of yeast broth cultures of bacteria is not likely to give false-positive agglutination results. We have also tested yeast broth cultures of many different kinds of bacteria against Type II antipneumococcus serum and normal and immune rabbit sera that do agglutinate yeast cells, and none of them have ever agglutinated the heterologous bacteria. Thus, control tests of the sterile broth itself against the agglutinating serum should eliminate any source of error in the agglutination of yeast broth cultures of bacteria.

The yeast broth grows pneumococci so well that it seemed particularly adapted for use in routine diagnostic typing. However, the fact that the yeast possesses some serological relationship to Type II pneumococci made it necessary to make many control experiments to determine whether or not false agglutination would occur with heterologous types of pneumococci grown in the yeast broth. In none of these experiments

was any heterologous type (Type I, III, IV) grown in yeast broth agglutinated or precipitated by Type II antiserum. From the result of these tests, pneumococcus cultures grown in yeast broth seem permissible for use in routine diagnostic typing, provided control tests are made to prove that the Type II serum does not precipitate the yeast broth medium itself.

USES OF THE YEAST MEDIA

The described yeast media have been used for some time in the clinical laboratory of bacteriology of the Hospital and have been found suitable for all routine procedures in which meat infusion peptone media are commonly used. The combined use of flasks containing 200 to 300 cc. of the "5 per cent yeast" broth and of blood plates from yeast agar have sufficed for the culture of most infectious material. The same "5 per cent yeast" broth and agar in deep tubes sealed with vaseline have been equally satisfactory in the culture of anaerobes from clinical material. Blood agar plates prepared from the "5 per cent yeast" medium have given uniform satisfaction, not only from the standpoint of nutrient properties but also from the standpoint of the properties (hemolysis and methemoglobin production) utilized in the differentiation of the Gram-positive cocci.

Although peptone is not added to yeast broth, it serves for the production of lysins (*B. Welchii*, tetanus bacillus and streptococcus) and toxins (diphtheria and tetanus). While not particularly interested in substituting the yeast broth for the usual meat infusion peptone medium in the production of toxin, we have regularly obtained more potent diphtheria toxin with the routine "5 per cent yeast" broth with all strains tested than under similar conditions with the best meat infusion peptone broth that could be prepared in this laboratory.

The "5 per cent yeast" broth has proved useful as a medium for pneumococcus cultures in typing. As a means of obtaining more prompt results in diagnostic typing, the "12 per cent yeast" broth has the advantage of more rapid growth. This medium, if inoculated with the heart's blood of a mouse, usually gives a culture of sufficient growth for typing and biling within 3 to 5

hours after inoculation. The "12 per cent yeast" broth, however, has the disadvantage of acid production below pH 6.0 if cultures are allowed to reach their maximum growth. In emergencies (which are the only times in which the "5 per cent" broth does not give sufficiently rapid growth) the cultures in "12 per cent" broth can be kept under constant observation and be tested before they become acid. (The necessity of controls against the possibility of serological reaction between the yeast broth and Type II antipneumococcus serum has been discussed under "Tests of Possible Serological Complications.")

COMMENT

A description has been given of the method of preparation of culture media from commercial dried yeast. Media prepared from yeast have been reported by other workers; in most cases, the medium has contained peptone or other substances in addition to the yeast; in other cases, the method of preparation has been somewhat too involved for a routine medium. Our object was to obtain a reliable culture medium, each batch of which could be depended upon for the growth of all kinds of bacteria that will grow in good batches, but not in poor batches, of meat infusion peptone media. At the same time, it was desired to keep the method simple enough to permit the preparation of the media in a reasonably short time by the ordinary technician. The described yeast media meet both these requirements and after a thorough test, we believe that they possess two definite advantages over meat infusion peptone media for all routine bacteriological procedures. The first advantage is reliability, in that each batch will support growth of pneumococci and other bacteria that fail to grow well in any batch of meat extract peptone broth or in a poor batch of meat infusion peptone media. Not a single one of 50 batches made from 4 different lots of the dried yeast has proved unsatisfactory. The second advantage is economy; the cost of the yeast and the phosphate is no greater than that of the peptone used in making meat infusion peptone media, and hence, the entire cost of the meat is saved. That the yeast media are as easy, or easier to prepare than meat infusion media, and that

they can be sterilized by a single process in the autoclave are factors that fit them for routine use.

Although the described yeast media have these definite advantages it is necessary to accept certain limitations to their use. The first is the production of acid by some bacteria. This complicates the use of yeast broth as a base for the special carbohydrate media employed in testing the fermenting properties of bacteria. However, in the usual laboratory, carbohydrate fermentation tests are used principally in the study of the colon-typhoid group of bacteria, which grow well in meat extract-peptone media. Consequently, we have made no attempt to use the yeast broth for this purpose, since we have considered it as a substitute for meat infusion peptone media and not for the cheap and easily prepared meat extract peptone broth. The second limitation is the possibility of complications arising from the fact that the yeast broth contains detectable amounts of the serologically reactive yeast carbohydrate described by Mueller and Tomcski (1924). However, the experiments carried out in this connection showed that errors are not likely to result from the routine use of yeast broth cultures in properly controlled agglutinination and precipitin tests. Nevertheless, it is probably inadvisable to use yeast broth cultures in immunological investigations (particularly in problems connected with pneumococci) until more is known of the serological relationships of yeast to bacteria.

SUMMARY

This paper describes the method of preparation of culture media from commercial dried yeast, and reviews their advantages and limitations. The "5 per cent yeast" broth and agar have been found satisfactory for all routine purposes for which meat infusion peptone media are commonly employed. The yeast media are easy to prepare and possess the definite advantages of uniform reliability and of low cost (9 cents per liter of broth). Their use is especially recommended for the routine culture of infectious material in clinical bacteriological laboratories.

REFERENCES

AVERY, O. T., AND HEIDELBERGER, M.: Jour. Exper. Med., 1925, **42**, 367.
MUELLER, J. H., AND TOMCSKI, J.: Jour. Exper. Med., 1924, **40**, 343.
SUGG, J. Y., AND NEILL, J. M.: Jour. Exper. Med., 1929, **49**, 183.

RELATIONSHIPS OF THE ENCAPSULATED BACILLI WITH SPECIAL REFERENCE TO BACT. AEROGENES[1]

PHILIP R. EDWARDS

From the Department of Veterinary Science, Kentucky Agricultural Experiment Station, Lexington, Kentucky

Received for publication November 19, 1928

The purpose of this paper is to present the results of work on the encapsulated bacilli and to discuss the relationships between some of the members of this group. This work is the outgrowth of a study of encapsulated bacilli found in cases of genital diseases in horses. These organisms have been described by Dimock and Snyder (1925), Dimock and Edwards (1927) and Edwards (1928). While these organisms were being compared with encapsulated bacilli isolated from human sources it was noted that certain cultures of *Bact. aerogenes* were closely related to the cultures from equine sources and to some of the cultures of human origin. This relationship seemed worthy of further study; so a number of cultures of *Bact. aerogenes* were collected and compared with the human and equine cultures in our possession.

SOURCE OF CULTURES

The cultures of Friedländer and granuloma bacilli which have been used in this work were obtained from several investigators who forwarded them to us. With one exception the strains of *Bact. aerogenes* were obtained in the same way. One of the cultures of *Bact. aerogenes* and the organisms from genital diseases in horses were isolated in this laboratory. Following are given the designations of the cultures and the sources from which they were obtained.

[1] Published with the approval of the director of the Kentucky Agricultural Experiment Station.

E1, E2, E8—isolated from uteri of infected mares F3, F5, F6, F8, F9, F10, F11, F13, F18, F20, F22, F23, F24, F25, F28, FR—Pneumonia, human
F1, F2—extirpated appendix, human
F21—extirpated tonsil, human
F26—chronic colitis, human
F27—chronic cystitis, human
F15—mitral regurgitation, human
F19—pleuritis, human
G2, G6—inguinal granuloma, human
AB—*Bact. aerogenes*, milk
AR, A9, A10, A14, AH9—*Bact. aerogenes*, feces
A3, A5, A7, A8, A11, A12, A50, A65, A70, A73, A97, A111, A112, A113, A114, A115, A153, AM8C—*Bact. aerogenes*, soil
A6, A13—*Bact. aerogenes*, water

The cultures E1, E2 and E8 represent a group of sixty-five encapsulated organisms recovered from cases of metritis in mares. Forty of these sixty-five strains have been studied and found identical in morphological, cultural, biochemical and serological properties. All are identical with type B of the Friedländer bacillus as described by Julianelle (1926). Since these cultures exhibit the same properties only three of them are included in this study.

CULTURAL CHARACTERISTICS

The cultural characteristics of the majority of the organisms included in this study are those generally attributed to the members of the *B. mucosus* group. An abundant, dirty white to slightly yellow growth which is moist, glistening and slimy occurs on agar slants. Agar colonies are large, raised, round and entire. The bacteria are Gram negative, non-sporing, non-motile rods which do not liquefy gelatin. Most of the organisms produce wide capsules on artificial media. Some of the cultures which have been grown on artificial media for long periods no longer produce the capsules typical of this group. Such strains do not exhibit a mucoid growth on agar and their virulence is reduced. This lack of virulence, loss of capsules and failure to exhibit a mucoid growth on agar occurred among all the groups studied,

the Friedländer, equine and granuloma strains as well as the cultures of *Bact. aerogenes* being affected in this way.

METHODS

The methods used in fermentation tests, immunization of rabbits, agglutination, agglutination absorption, and precipitin tests are those employed by Edwards (1928). The protein antigens used in the precipitin tests were prepared by the method of Julianelle (1926b).

TABLE 1

Biochemical reactions

	AMMONIA PRODUCTION	NITRATE PRODUCTION	INDOL PRODUCTION	METHYL RED	VOGES-PROSKAUER	GROWTH IN URIC ACID	GROWTH IN CITRATE	ACTION IN LITMUS MILK
E1, E2, E8, F6, F26, A3, F11, A5, A7, A10, A13, A50, A65, A113, A115, A150, A153 AB, AH9, G6.	+	+	−	−	+	+	+	AC
F3, F5, F9, F15, F18, F19, F23, F25, F28, A6, A11, A14, A114	+	+	−	−	+	+	+	A
F2, F8, F10, F24, F27, FSc.	+	+	−	+	−	+	+	A
F21, A97, A112	+	+	−	+	−	+	+	AC
F1	+	+	−	+	−	−	+	A
F13, FR	+	+	−	+	−	−	−	A
F20	+	+	−	−	+	−	−	A
F22	+	+	−	−	+	−	−	Al
AR, A8, A9, A111	+	+	+	−	+	+	+	AC
A73, AM8C	+	+	+	−	+	+	+	A
A70	+	+	+	+	−	+	+	A
A12	+	+	−	+	−	+	−	A
G2	+	+	−	−	−	+	+	AC

BIOCHEMICAL REACTIONS

The cultures studied have been subjected to the tests usually applied to the organisms of the colon-aerogenes group. The strains have been tested for indol production, growth in citrate and uric acid media, nitrate reduction, production of ammonia, action in litmus milk and response to the methyl red and Voges-Proskauer tests.

TABLE 2

Fermentative reactions

	GLUCOSE	LEVULOSE	GALACTOSE	LACTOSE	SUCROSE	GLYCEROL	DULCITOL	MANNITOL	SORBITOL	ARABINOSE	XYLOSE	RAFFINOSE	MALTOSE	ADONITOL	SALICIN	INULIN	DEXTRIN	SOLUBLE STARCH
E1, E2, E3, F3, F5, F8, F6, F13, F15, F19, F20, F21, F24, F25, F26, F28, AB, A3, A13, A14, A50, A112, A113, A114, G2	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F2	AG	AG	AG	AG	O	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F23	AG	AG	AG	O	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
F10, F27, A150, AH9	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
Sc	A	A	A	A	A	A	A	AG	AG	A	A	A	A	A	A	O	AG	AG
F1	A	A	A	A	O	A	O	A	A	A	A	A	A	A	A	O	A	A
F11	A	A	A	A	A	O	O	A	O	A	A	O	O	O	O	O	O	O
F18	A	A	A	A	A	A	O	A	A	A	A	A	A	A	A	O	A	A
F22	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
G6	A	AG	A	AG	A	A	O	A	A	A	A	A	A	AG	A	O	AG	AG
AR, A6, A9, A97	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
A5, A7	AG	AG	AG	AG	AG	O	O	AG	AG	AG	AG	AG	AG	O	AG	O	AG	AG
A10	AG	AG	AG	A	AG	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	O	AG	AG
A11, A150, AH9	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
A12	AG	AG	AG	AG	AG	O	O	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	O
A65	AG	AG	AG	AG	AG	AG	O	AG	AG	AG	AG	AG	AG	O	AG	O	AG	AG
A115	AG	AG	AG	AG	AG	O	O	AG	AG	AG	AG	AG	AG	AG	AG	O	AG	AG
A153	AG	AG	AG	AG	AG	O	O	AG	AG	AG	AG	AG	AG	O	AG	O	AG	O
A8, A70, A73, A111	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG

From table 1 it can be seen that the organisms studied are divisible into several groups on the basis of their reactions to the tests employed. These groups do not appear to be significant however, since members of the various species are dispersed through several groups. It is evident that this combination of tests cannot be employed in the separation of the various species. If the results of each of the tests be considered separately it may be seen that not one of the tests employed can be used to differentiate the various species.

FERMENTATIVE REACTIONS

The cultures studied were tested for their ability to form acid and gas from a number of fermentable substances. An examination of table 2 shows that the cultures of the various types cannot be separated by their action upon the fermentable substances used. Certain groups are established by the fermentation tests but the various species are dispersed throughout these groups. The groups established by the fermentation reactions do not agree with those established by the biochemical reactions given in table 1, nor, as will be shown later, are they related to the groups established by the serological tests. Both the cultures of *Bact. aerogenes* and the Friedländer bacilli are rather variable in their fermentative characters.

SEROLOGICAL REACTIONS

The early literature regarding the serological properties of the Friedländer group is very confusing. Later workers have successfully classified the organisms by serological methods. Toenniessen (1914) suggested that the capsular substance was antigenic in character and that Friedländer bacilli were capable of exhibiting two antigenic complexes, one residing in the capsule and the other in the endoplasm of the bacterium. Toenniessen (1921) later demonstrated that the capsule of these organisms was a galactan. Heidelberger, Goebel and Avery (1925) derived a nitrogen-free polysaccharide from Friedländer bacilli which was capable of giving positive precipitin tests with homologous immune serum. Julianelle (1926, 1926a, 1926b) by applying

these facts to the classification of Friedländer strains has established four serological groups among the encapsulated bacilli. These he has designated as types A, B, and C, and Group X. Types A, B and C are specific while group X is composed of a number of heterologous strains.

Julianelle demonstrated that organisms which produced capsules were agglutinated by serum derived from an organism of the homologous type but were not affected by sera derived from organisms of heterologous types. The manner in which these organisms were clumped was characteristic, agglutination resulting in the formation of a compact, voluminous disc at the bottom of the tube. Organisms which no longer produced capsules, or bacilli which had been stripped of their capsule by chemical methods, did not exhibit this type specificity. They were agglutinated by the immune sera of both the homologous and heterologous types. The character of the agglutination was changed. Instead of the formation of a compact disc, agglutination resulted in a fine, powdery precipitate.

In the present investigation the Friedländer, granuloma, and equine organisms have been compared with the cultures of *Bact. aerogenes* using agglutination, precipitin, and agglutinin absorption tests in an effort to determine their relationships.

AGGLUTINATION

Cultures E1, F6, FSc, G2, G6, and A3 were used to prepare antisera. Using these sera, agglutination tests were set up in dilutions ranging from 1 to 20 to 1 to 2000. Two main types are established by the agglutination tests. The first group composed of strains E1, E2, E8, F6, F26, FR, AB, A3, A13, A113, and A114 is identical with type B of the Friedländer bacillus as described by Julianelle (1926). These organisms have the cultural characters generally attributed to the soil types of *Bact. aerogenes*. It will be noted that in this type are included five strains of *Bact. aerogenes*, three isolated from soil, one from water and one from milk.

The second large group is composed of strains F3, F5, F8, F9, F18, F19, F20, F21, F23, F24, F25, F28, FSc. These strains are identical with type A of the Julianelle.

A third group established by the agglutination test is composed of three cultures, G6, A70, and A73. G6 is a culture received from Dr. J. C. Small. It was recovered from a local lesion of inguinal granuloma. The cultures A70 and A73 are soil strains of *Bact. aerogenes* received from Dr. S. A. Koser.

In addition to the specific agglutination observed in these tests several instances of group or non-specific agglutination occurred among the aerogenes strains. This was most evident in the cases of strains A12 and A14. Julianelle (1926a) attributed group agglutination among the Friedländer bacilli to the absence of capsule formation. The same cause is probably operative in the case of *Bact. aerogenes*. The cultures which were acted upon by the group agglutinin exhibited a rather dry growth on agar and no capsules could be demonstrated in these cultures.

During the course of this work the action of hydrolysis upon agglutination of the encapsulated bacilli has been studied. This method, first used by Porges (1905), and since employed with more or less success by a number of investigators, consists of treating suspensions of encapsulated bacilli with one fourth volume of 0.25 N HCl, heating at 80°C. for fifteen to thirty minutes and neutralizing the suspension by the addition of NaOH. This treatment is designed to strip the organism of its capsule and render it easily agglutinable. Beham (1911) stated that this method often rendered the bacilli spontaneously agglutinable. The results of our work with hydrolyzed suspensions have, on the whole, been unsatisfactory. Many organisms thus treated became spontaneously agglutinable. Still other strains, after they had been so treated, were not affected by sera known to be potent in group agglutinins.

However, by varying the temperature and the time of exposure to acid we have been able to render a number of the strains susceptible to group agglutinin. We have not been able to distinguish *Bact. aerogenes* from the other types by this method. We have found that, after hydrolysis, *Bact. aerogenes* strains are agglutinated by Friedländer antisera to about the same extent as the equine organisms and Friedländer and granuloma bacilli.

PRECIPITIN TESTS

The tests performed with type specific precipitin antigen confirm the results of the agglutination tests in every case. The cultures which were agglutinated by sera derived from strains E1 and F6 gave strongly positive precipitin tests with these sera while no precipitation resulted with sera derived from strains G6 and Sc. Also, strains A70 and A73 gave strongly positive tests with serum derived from strain G6 but caused no precipitation when placed in contact with the sera of other types. The strains composing type A of Julianelle were also specific in their precipitin reactions.

In the precipitin tests using protein antigens the proteins derived from strains of *Bact. aerogenes* yielded strongly positive tests with antisera derived from cultures of Friedländer and granuloma bacilli. It was not possible to distinguish between the organisms of the various types in this way. Julianelle (1926b) has observed the precipitation occurring when Friedländer antisera are placed in contact with protein derived from *Bact. aerogenes*. This reaction indicates the close serological relationship of the proteins of these bacilli.

AGGLUTININ ABSORPTION

The results of the agglutinin absorption tests further substantiate the relationships established by the agglutination tests and precipitin tests. In every case in which type specific agglutination occurred the strain so agglutinated was able to exhaust the type serum of agglutinins completely. Thus, strains E2, E8, F6, F26, FR, AB, A3, A13, A113, and A114 effected a complete removal of agglutinins active on strain E1 from antiserum derived from that strain. They were also able completely to remove the agglutinins active on strain F6 from antiserum derived from F6. Further, these strains caused a complete removal of agglutinins from A3 antiserum. This group of organisms was inactive when used to absorb the sera of other types.

Similarly strains A70 and A73 completely exhausted antiserum G6 of agglutinins for the serum strain. They were without

effect when used to absorb antisera derived from strains E1 and FSc.

The group of Friedländer strains composing type A were also apparently of homogeneous antigenic structure since they all exhausted serum FSc of agglutinins active upon the homologous strain.

The strains which were agglutinated non-specifically were unable to completely remove agglutinins from any of the antisera.

PATHOGENICITY

Baerthlein (1918) and Toenniessen (1921) have demonstrated that the virulence of Friedländer bacilli for the laboratory animals is dependent upon capsule formation. In a previous publication, Edwards (1928), we have found that the ability of organisms of this group to cause metritis in mares is also dependent upon abundant capsule formation.

In studying the effect of cultures of *Bact. aerogenes* on laboratory animals we have found that those cultures which produced wide capsules and a mucoid growth on artificial media were uniformly virulent for the laboratory animals. Using the technique described by Dimock and Edwards (1926), it has been found possible to produce metritis in mares through the introduction of *Bact. aerogenes* into the uterus. However, only those strains which were vigorous capsule producers were able to become established.

No constant differences in pathogenicity of the various types have been observed.

DISCUSSION

It is evident from the results obtained in this study and from the results of other workers that such tests as ammonia production, nitrate reduction, indol formation, growth in uric acid and citrate media, and the methy red and Voges-Proskauer tests are of no value in the differentiation of the various types of encapsulated bacilli.

The action of the organisms when grown in milk has been employed by many writers to set apart *Bact. aerogenes* from the

other members of the group. Basing their statements on the work of Wilde (1896) and Claremont (1902) they characterize *Bact. aerogenes* as a milk coagulator, while Friedländer's bacillus is described as causing no coagulation in milk. However, the results of other workers have been contradictory. Fricke (quoted by Abel and Hallwacks (1913) found a striking variation in the ability of strains isolated from pathological sources to coagulate milk. Fitzgerald (1914) observed that certain strains of Friedländer's bacillus were able to coagulate milk while other strains caused no coagulation. Even when the utmost care was used in the tests, these differences were apparent and Fitzgerald warns against the use of coagulation of milk as a differential test. Small and Julianelle (1923) studied 13 cultures of Friedländer bacilli and found that 5 of the strains coagulated milk.

In the present study we have found that four strains of Friedländer bacilli cause a coagulation in milk. It is noteworthy that two of the Friedländer strains used in this study which did not produce coagulation in milk had been previously studied by Small and Julianelle and found to be milk coagulators. Among the aerogenes cultures studied there were seven which caused a permanent acidity in milk but produced no coagulation. Others coagulated the milk only after four to ten days incubation. In addition it must be remembered that 40 cultures of equine origin, which are identical with type B of the Friedländer bacillus, rapidly coagulate milk. In view of these facts it would seem that coagulation of milk must be accepted with reservations, if it is accepted at all, as a means of differentiating *Bact. aerogenes* from the other members of the encapsulated group.

The fermentative characters of the encapsulated bacilli have been studied by a number of workers and several classifications have been based upon the action of these organisms on fermentable substances. These studies, however, are far from being in agreement. The greatest controversy has centered about the ability of Friedländer's bacillus to ferment lactose. Many writers contend that this organism produces acid and gas from glucose and sucrose but does not attack lactose. Friedländer (1882, 1883, 1884) did not mention the action of the organisms

which he isolated on milk or lactose. Strong (1889) working with strains which were presumably lineal descendants of Friedländer's original cultures found that the organism produced no acid or gas from lactose. Grimbert (1895) stated that Friedländer's bacillus was able to produce acid from lactose. Wilde (1896) reported that the strains of Friedländer bacilli which he studied formed acid from lactose. Nicolle and Hebert (1897) found that Friedländer's bacillus formed acid and gas from lactose. Russ (quoted by Abel and Hallwachs (1913) stated that Friedländer's bacillus fermented lactose. Lehman and Neumann (1901) reported that Friedländer's bacillus fermented lactose with the production of acid and gas. Claremont (1902) found that all of the ten Friedländer strains which he studied produced acid from lactose. Nine of these strains formed perceptible gas. Perkins (1904) classified the encapsulated bacilli according to their action on glucose, lactose, and sucrose. Those strains which were able to ferment glucose and sucrose but did not attack lactose he designated as Friedländer's bacillus. A culture from Kral, supposedly a descendant of the original strain of Friedländer, did not attack lactose. MacConkey (1905) on the contrary, found that a transplant obtained indirectly from the Kral culture produced acid in lactose broth. Fitzgerald (1914) found that six of seven Friedländer strains examined attacked lactose and offered evidence that fermentation could not be relied upon in the classification of the encapsulated bacilli. Coulter (1917) studied eleven strains of Friedländer bacilli and found that none of them fermented lactose and that they formed a uniform serological group. Castellani and Chalmers (1919) in their classification of the colon-typhoid group inserted the genus Encapsulatus. The type species, *Encapsulatus pneumoniae*, Friedländer 1883, was described as forming acid and gas from glucose and sucrose and acid alone from lactose. *Encapsulatus aerogenes* was said to ferment glucose, lactose, and sucrose with the formation of acid and gas. Bergey (1921) stated that Friedländer's bacillus formed acid and gas from lactose. Small and Julianelle (1923) found that eleven of thirteen Friedländer strains which they studied produced acid from lactose. In addition five of the cultures which

they studied produced gas from this sugar. Perkins (1925) supplementing his former work, again classified the Friedländer bacillus as a non-lactose fermenter.

In this work we have studied twenty-two strains of Friedländer bacilli. With the exception of three strains, all of these organisms formed acid from lactose and, in addition, sixteen of the twenty-two cultures formed a perceptible amount of gas.

The conflicting results obtained by various workers, each believing himself to be working with the original strain of Friedländer's bacillus, are worthy of note. It seems from a review of the literature that most of the cultures of excapsulated bacilli isolated from cases of pneumonia and from the respiratory tract are able to ferment lactose. If lactose fermentation is used as a criterion for the differentiation of species it will result in the separation of organisms serologically identical into different species. Such a procedure hardly seems justified when the majority of the organisms labelled as Friedländer's bacillus are lactose fermenters.

The agglutination of aerogenes strains by Friedländer sera was noted by Claremont (1902). However, the same strains of *Bact. aerogenes* when used to prepare serum did not agglutinate any of his strains of Friedländer bacilli. It is highly probable that this agglutination was non-specific or group agglutination. Bertarelli (1906) also has observed the agglutination of aerogenes by Friedländer sera. Julianelle (1926a) calls attention to the close relationships of aerogenes and Friedländer proteins. He explains the non-specific agglutination of capsule free strains of *Bact. aerogenes* on the basis of this relationship between the proteins of the two organisms.

It is the opinion of the writer that specific agglutination occurring between Friedländer's bacillus and *Bact. aerogenes* has not been heretofore recorded. Tomcsik (1927) has isolated a protein free carbohydrate-like specific substance from strains of *Bact. aerogenes*. This substance, however, did not prove reactive with anti-Friedländer sera.

In the present work it has been found that three strains of *Bact. aerogenes* isolated from soil, one strain isolated from water

and one strain isolated from milk are identical with type B of the Friedländer bacillus as described by Julianelle (1926). Two other strains of *Bact. aerogenes* have been found to be antigentically identical with a strain of the granuloma organism. Sera for only three types of the encapsulated bacilli were used in this study. If sera were prepared for the other types of Friedländer bacillus and the strains of *Bact. aerogenes* tested with these, it is probable that still further relationships would be established. Using only the three type sera, it has been found that seven of 26 cultures of *Bact. aerogenes* are identical in their antigenic structure with certain of the Friedländer and granuloma bacilli.

It will be noted that no aerogenes cultures were found to be identical with type A of the Friedländer bacillus. This type, apparently, is most commonly found in human infections, while type B seems to be found most frequently in the lower animals.

Bergey (1921) has classified *Bact. aerogenes* under the genus Aerobacter while Friedländer's bacillus is classified under the genus Encapsulatus. Perkins (1925) includes both of these organisms in the same genus, Encapsulata, and differentiates them on the basis of lactose fermentation. Weldin (1927) places *Bact. aerogenes* in the genus Aerobacter and places Friedländer's bacillus in the genus Proteus. Castellani and Chalmers (1919) place the two organisms in the same genus, Encapsulatus, and differentiate them by the formation of gas from lactose.

From the results obtained in this study it seems that *Bact. aerogenes* is so closely related to Friedländer's bacillus and the bacilli isolated from lesions of inguinal granuloma that it should certainly be placed in the same genus as the remainder of the encapsulated bacilli. There are no constant differences between the strains which we have received labelled as Friedländer's bacilli and *Bact. aerogenes*. Furthermore we have found no distinguishing characters which might be used to separate these organisms into two or more species.

CONCLUSIONS

1. The organisms which we have received from various sources labelled Friedländer bacilli cannot be distinguished from *Bact.*

aerogenes and the other members of the encapsulated group by action on milk or fermentative characters.

2. Five cultures of *Bact. aerogenes* isolated from soil, water and milk have been found to be culturally, biochemically, and serologically identical with type B of the Friedländer bacillus as described by Julianelle. Two cultures of *Bact. aerogenes* have been found serologically identical with a strain of the granuloma bacillus.

3. *Bact. aerogenes* is so closely related to the other encapsulated forms that they should be classified in the same genus. No constant differences have been observed which could be used to separate the organisms into two or more species.

REFERENCES

ABEL, R., AND HALLWACHS, W. 1913 Die Kapselbacillen. Kolle und Wassermann: Handbuch der Pathogenen Mikroorganismen. Jena, 1913, **4**, 515–544.

BAERTHLEIN, K. 1918 Ueber bacterielle Variabilität inbesondere so-genannte Bakterienmutationen. Centralbl. f. Bakt., I, Orig., **81**, 369.

BEHAM, L. M. 1911 Die agglutinatorischen Eigenschaften der Kapselbacillen und die Anwendung der Serumagglutination bei den Trägern von Kapselbacillen. Centralbl. f. Bakt., I, Orig., **66**, 110.

BERGEY, DAVID H., ET AL. 1923 Manual of Determinative Bacteriology. Williams & Wilkins Co., Baltimore.

BERTARELLI, E. 1906 Centralbl. f. Bakt., I, Ref., **37**, 343.

CASTELLANI, A., AND CHALMERS, A. J. 1920 Sur la classification de certains groupes de bacilles aérobies de le intestine humain. Ann. Inst. Past., **34**, 600–621.

CLAREMONT, P. 1902 Differentialdiagnostische Untersuchungen über Kapselbakterien. Zeitschr. f. Hyg. und Infektionkr., **39**, 1–85.

COULTER, C. B. 1917 The biological entity of the Friedländer bacillus. Jour. Exper. Med., **26**, 763.

DIMOCK, W. W., AND EDWARDS, P. R. 1927 Genital infection in mares by an organism of the Encapsulatus group. Jour. Amer. Vet. Med. Assoc., **70**, 469–480.

DIMOCK, W. W., AND SNYDER, E. M. 1924 Sterility in mares. Thirty-seventh annual report of the Kentucky Agricultural Experiment Station, p. 18.

EDWARDS, P. R. 1928 The relationship of encapsulated bacilli found in metritis in mares to encapsulated bacilli from human sources. Jour. Bacteriol., **15**, 245–266.

FITZGERALD, J. G. 1914 A biochemical study of the mucosus-capsulatus group. Jour. Inf. Dis., **15**, 268–278.

FRIEDLÄNDER, C. 1882 Ueber die Schizomyceten bei der acuten fibrösen Pneumonie. Virchow's Arch. f. Path. Anat., **87**, 319–324.

FRIEDLÄNDER, C. 1883 Die Mikrokokken der Pneumonie. Fortschr. d. Med., **1**, 715–733.

FRIEDLÄNDER, C. 1884 Weitere Bemerkungen über Pneumonie Mikrokokken. Fortschritte der Med., **2**, 333–336.

GRIMBERT, L. 1895 Recherches sur le pneumobacille de Friedländer. Premier mémoire, Étude des fermentations provoquée par cet organisme. Ann. Inst. Past., **9**, 840.

HEIDELBERGER, M., GOEBEL, W. F., AND AVERY, O. T. 1925 The soluble specific substance of a strain of Friedländer's bacillus. Jour. Exper. Med., **42**, 701–707.

JULIANELLE, L. A. 1926 A biological classification of *Encapsulatus pneumoniae* (Friedländer's bacillus). Jour. Exper. Med., **44**, 113–128.

JULIANELLE, L. A. 1926a Immunological relationships of encapsulated and capsule-free strains of *Encapsulatus pneumoniae* (Friedländer's bacillus). Jour. Exper. Med., **44**, 683–696.

JULIANELLE, L. A. 1926b Immunological relationships of cell constituents of *Encapsulatus pneumoniae* (Friedländer's bacillus). Jour. Exper. Med., **44**, 735–751.

LEHMANN, K. B., AND NEUMANN, R. 1901 Atlas and Principles of Bacteriology, 2nd edition, Part II. English Translation. W. B. Saunders & Co., Philadelphia.

MACCONKEY, A. 1905 Lactose fermenting bacteria in feces. Jour. Hyg., **5**, 333.

NICOLLE, C., AND HEBERT, A. 1897 Note sur un enchantillon de bacille de Friedländer. Ann. Inst. Past., **11**, 80.

PERKINS, R. G. 1904 *Bacillus mucosus capsulatus*. A study of the groups and an attempt at classification of the varieties described. Jour. Inf. Dis., **1**, 241–266.

PERKINS, R. G. 1925 Classification of spore-free, Gram-negative, aerobic rods with special reference to fermentation and proteolysis. Jour. Inf. Dis., **37**, 232–255

PORGES, O. 1905 Ueber die Agglutinabilität der Kapselbakterien. Wien. Klin. Wchnschr., **18**, 691–693.

STRONG, L. W. 1889 Ueber die Kapselbacillen. Centralbl. f. Bakteriol., I, **25**, 49.

TOENNIESSEN, E. 1914 Ueber die Agglutination der Kapselbacillen. Centralbl. f. Bakt., I, Orig., **75**, 329–336.

TOENNIESSEN, E. 1921 Untersuchungen uber die Kapsel (Gummihülle) der pathogenen Bakterien. Centralbl. f. Bakt., I, Orig., **85**, 225–237.

TOENNIESSEN, E. 1921a Ueber die Variationsformen der Bakterien und ihre Uebereinstimmung mit den Variationsformen der Metazoen. Centralbl. f. Bakt., I, Orig., **86**, 353–380.

TOMCSIK, J. 1927 On carbohydrate-like specific substance in the colon-aerogenes group. Proc. Soc. Exper. Biol. and Med., **24**, 810.

WELDIN, J. C. 1927 The colon typhoid group of bacteria and related forms. Iowa State Col. Jour. Sci., **1**, 121–197.

WILDE 1896 Centralbl. f. Bakt., I, Ref., **20**, 681.

PRELIMINARY REPORT ON A METHOD FOR THE DIFERENTIATION OF L. ACIDOPHILUS FROM L. BULGARICUS

WALTER L. KULP

Department of General Bacteriology, Yale University

Received for publication November 21, 1928

At the present time there appears to be much confusion in the classification and identification of the lactobacilli, particularly with reference to *L. acidophilus* and *L. bulgaricus*. During recent years these two species have received a considerable amount of study from research workers in this field, and several tests have been advocated as aids in the identification of typical strains of these bacteria.

Morphologically and culturally. *L. acidophilus* and *L. bulgaricus* are quite similar; however, there exist marked physiological differences between them. Strains of true *L. bulgaricus* do not attack maltose, sucrose, and unheated levulose (Kulp and Rettger, 1924) while all strains of true *L. acidophilus* are able to ferment these sugars, producing acid without gas. The first named species will live in the intestine of man and warm-blooded animals when these subjects are given a diet which is high in lactose or dextrin content. On the contrary, under similar conditions *L. bulgaricus* is killed by passage through the digestive tract (Hull and Rettger, 1914; Rahe, 1915; Kulp 1926). Albus and Holm (1926), and, since then, Kopeloff and Beerman (1927), have reported that *L. acidophilus* will grow at lower surface tensions than will *L. bulgaricus*. Day and Gibbs (1928) disagree with the conclusions that these species may be differentiated from each other by surface tension methods, and claim that whatever differences are obtained may be ascribed to varying toxic action.

Unfortunately for the taxonomist and laboratory worker, there are members of this genus which appear to lie midway

between typical *L. acidophilus* and typical *L. bulgaricus*. The writer has had occasion to study several of these so-called border-line strains which morphologically and culturally are similar to *L. acidophilus* and *L. bulgaricus*, although growth in culture media is more profuse than that of either of these two species. This increased growth constitutes a difference in degree only and, therefore, should not be given much consideration as a differential characteristic. Without exception, the border-line organisms which have been studied by the writer have failed to survive passage through the digestive tract. So-called surface tension experiments classify them with *L. bulgaricus*, leaving small doubt that they are closely related variants of this species.

Within recent years some investigators have designated an organism associated with dental caries and, in rare instances, isolated from intestinal contents, as *L. acidophilus*. Evidence at hand establishes a firm conviction that this organism or group of organisms is wrongly named. Research by Morishita (1928) in our laboratory clearly indicates that the type usually associated with dental caries is not *L. acidophilus* (Moro). The present report does not deal with the dental caries organism or organisms, but mention is made of them here because of their close relation to the subject under discussion and because of the interest they have aroused. Any trained bacteriologist can easily recognize differences between the type of organism commonly associated with caries and representative strains of *L. acidophilus* or *L. bulgaricus*.

Because of the therapeutic properties attributed to *L. acidophilus* by Torrey (1915), Rettger and Cheplin (1921), Kopeloff (1924) and others, the separation of authentic strains of *L. acidophilus* from *L. bulgaricus* types by control laboratories and research workers has assumed much importance, and additional methods of identification appear highly desirable.

In the course of the present investigation it was found that *L. acidophilus* had a decidedly greater tolerance for indol and phenol than did *L. bulgaricus*. A more detailed study revealed that the degree of tolerance could be employed for differential purposes.

Five strains of representative *L. bulgaricus*, two of the maltose-attacking variants of *L. bulgaricus* and five strains of typical *L. acidophilus* were employed in these experiments. A summary of their more important characteristics is given in table 1.

Casein digest made from chemically pure casein containing no carbohydrate was employed as the nitrogenous base for all sugar broths. The inoculum was prepared by centrifuging

TABLE 1

Important characteristics of L. acidophilus and L. bulgaricus strains employed in these experiments

	LACTOSE	MALTOSE	GALACTOSE	UNHEATED LEVULOSE	SUCROSE	INULIN	COLONY TYPE	NO GROWTH AT
								°C.
L. bulgaricus:								
B1	a	0	a	0	0	0	x	20
B2	a	0	a	0	0	0	x	20
B3	a	0	a	0	0	0	x	20
B4	a	0	a	0	0	0	x	20
B6	a	a	a	0	0	0	x	18
B7	a	a	a	0	0	0	x	14
L. acidophilus:								
AH1 (human)	a	a	a	a	a	a	x	20
AH2 (human)	a	a	a	a	a	a	x	20
AH3 (human)	a	a	a	a	a	a	x	20
RH1 (rat)	a	a	a	a	a	a	x and y	20
RH2 (rat)	a	a	a	a	a	a	x and y	20

a = growth and acid production.
0 = no growth.
x and y types of colony (Rettger and Horton, 1914).

forty-eight-hour glucose broth cultures and suspending the sedimented organisms in sterile saline. Broth cultures were incubated for four days at 37°C. Acidity was ascertained by the colorimetric method of pH determination. A decrease of 0.5 on the pH scale lower than the pH of the control mixture (carbohydrate-free casein digest broth and inoculum) was considered as evidence of fermentation. Visible growth was absent in broth

cultures which showed no decrease in the pH. Limiting growth temperature was determined by incubating inoculated glucose broth tubes for seven days.

When levulose is employed in fermentation studies the following precautions should be taken, in order to secure uniform results. Secure the purest sugar obtainable; sterilize a concentrated solution by filtration, and add, aseptically, the necessary amount to each tube of sterilized broth (Kulp and Rettger, 1924).

The X type colony is, in our opinion, a significant characteristic of *L. acidophilus* or *L. bulgaricus*. All isolations of *L. acidophilus* employed in this study were made from typical X type colonies. Strains of true *L. bulgaricus* should produce colonies of a similar type.

Day and Gibbs (1928) state, as their belief, that fermentation reactions cannot be employed to differentiate *L. acidophilus* from *L. bulgaricus*. The writer's results are in disagreement with their claim. He has not been able to secure any culture of typical *L. bulgaricus* which will ferment maltose, sucrose, or levulose. A few strains of border-line organisms have produced acid from maltose, but these types did not attack sucrose or levulose.

Any satisfactory plating medium for *L. acidophilus* or *L. bulgaricus* may be employed in the determination of the tolerance of these species for indol or phenol. Tomato peptone agar (Kulp, 1927) and casein-digest galactose agar (Kulp, 1924) were chosen for these experiments. It appears that the latter is slightly more desirable as a medium for *L. bulgaricus* than the former.

The agar was distributed and sterilized in test tubes, 10 cc. to each tube. The indol was prepared as a 10 per cent alcoholic solution, and the phenol as a 5 per cent aqueous solution; these solutions are self-sterilizing. Cultures in milk which had been incubated at 37°C. for twenty-four hours were employed as the inoculum. Platings containing no indol or phenol were prepared as controls. One cubic centimeter of 1:10,000 dilution of the culture was placed in a sterile Petri dish. The desired amount of indol or phenol solution was added to the melted agar in the tube

and well mixed, after which the agar was added to the culture in the plate. Indol agar plates, phenol agar plates, and the control plates were incubated in separate containers in order to eliminate the effect of volatilized indol or phenol. All cultures were incubated for three days at 37°C. in an atmosphere containing approximately 5 per cent CO_2 (Kulp, 1926).

The inhibitive effect was revealed by the absence of colony development visible to the naked eye. All controls developed an abundant growth.

In later experiments Petri dishes were eliminated. The culture and the desired dilutions of indol and phenol were added to a measured amount of melted agar in test tubes. After mixing thoroughly the agar was allowed to solidify. The cultures thus prepared were placed in tins. After sufficient CO_2 had been added to these tins to constitute about 5 per cent of the atmospheric volume, they were tightly sealed and incubated at 37°C for three days.

Either of the above methods is satisfactory. However, the second is preferable because of its greater simplicity.

The results of representative experiments are indicated in tables 2 and 3.

An experiment was completed which determined the tolerance of several strains of *L. acidophilus* and *L. bulgaricus* for given concentrations of indol and phenol over exposure periods of considerable length.

One-tenth of a cubic centimeter of a twenty-four hour culture grown in milk was added to a tube of melted tomato agar (0.4 per cent agar), which was held at 37°C. to prevent solidification. Five-hundredths of a cubic centimeter of a 10 per cent indol solution was mixed with the inoculated agar; similarly 0.5 cc. of a 5 per cent phenol solution was added to another melted and inoculated agar tube. Transfers were made with a 4 mm. loop from each of these cultures into sterile agar at one, two and twenty-four hour intervals. It was discovered that there was a slight decrease in viable organisms of both species after one and two hour exposures. After an exposure of twenty-four hours the *L. bulgaricus* cultures were found to have been killed; on the other hand, the *L. acidophilus* cultures contained many viable cells.

TABLE 2

Growth in graded concentrations of indol

STRAIN	1:3300	1:2500	1:2000	1:1650	1:1450	1:1250	1:1100	1:1000	CONTROL
L. bulgaricus:									
B1	+	0	0	0	0	0	0	0	4+
B2	+	0	0	0	0	0	0	0	3+
B3	+	0	0	0	0	0	0	0	2+
B4	+	0	0	0	0	0	0	0	4+
B5	3+	+	0	0	0	0	0	0	4+
B6	+	0	0	0	0	0	0	0	3+
B7	+	0	0	0	0	0	0	0	3+
L. acidophilus:									
AH1	4+	4+	4+	4+	3+	2+	±	0	4+
AH2	4+	4+	4+	4+	4+	3+	+	0	4+
AH3	4+	4+	4+	4+	4+	3+	+	0	4+
AR1	4+	4+	4+	4+	4+	3+	2+	1	4+
AR2	4+	4+	4+	4+	4+	3+	±	±	4+

+ = visible growth.
4+ = very heavy growth.
± = barely visible growth.

TABLE 3

Growth in graded concentrations of phenol

STRAIN	1:750	1:500	1:400	1:300	1:275	1:250	1:225	1:200	CONTROL
L. bulgaricus:									
B1	2+	+	0	0	0	0	0	0	4+
B2	2+	+	0	0	0	0	0	0	3+
B 3	2+	+	0	0	0	0	0	0	2+
B4	2+	+	+	0	0	0	0	0	4+
B5	2+	+	0	0	0	0	0	0	4+
B6	2+	+	+	0	0	0	0	0	3+
B7	2+	+	0	0	0	0	0	0	3+
L. acidophilus:									
AH1	4+	2+	+	+	±	0	0	0	4+
AH2	4+	3+	2+	2+	+	±	0	0	4+
AH3	4+	3+	3+	3+	+	±	0	0	4+
RH1	4+	4+	4+	4+	4+	3+	±	±	4+
RH2	4+	4+	3+	3+	2+	+	+	±	4+

+ = visible growth.
4+ = very heavy growth.
± = barely visible growth.

The results of these experiments indicate that strains of typical *L. acidophilus* can be separated from strains of representative *L. bulgaricus* by the determination of their tolerance for indol or phenol. The very slight tolerance of *L. bulgaricus* for indol and phenol *in vitro* experiments may help to explain why *L. bulgaricus* is not able to survive passage through the digestive tract. Further studies are planned to determine the validity of this theory.

REFERENCES

ALBUS, W. R., AND HOLM, G. E. 1926 The effect of surface tension upon the growth of *L. acidophilus* and *L. bulgaricus*. Jour. Bact., **12**, 13–18.

DAY, A. A., AND GIBBS, W. M. 1928 Surface tension in relation to bacterial growth, with special reference to *L. acidophilus* and *L. bulgaricus*. Jour. Infect. Dis., **43**, 97–107.

HULL, T. G., AND RETTGER, L. F. 1914 The influence of milk and carbohydrate feeding on the intestinal flora of white rats. Centralbl. f. Bakteriol., 1 Abt., Orig., **75**, 219–229.

KOPELOFF, N. 1924 Clinical results obtained with *Bacillus acidophilus*. Arch. Int. Med., **33**, 47–54.

KOPELOFF, N., AND BEERMAN, P. 1927 Surface tension studies with *L. acidophilus* and *L. bulgaricus*. Jour. Infect. Dis., **40**, 656–666.

KULP, W. L., AND RETTGER, L. F. 1924 Comparative study of *L. acidophilus* and *L. bulgaricus*. Jour. Bact., **9**, 357–394.

KULP, W. L. 1926a Viability of *B. acidophilus* and *B. bulgaricus* in the human intestine. Jour. Amer. Med. Assoc., **87**, 833–835.

KULP, W. L. 1926b The determination of viable *L. acidophilus*. Science, **64**, 304–306.

KULP, W. L. 1927 An agar medium for plating *L. acidophilus* and *L. bulgaricus*. Science, **66**, 512–513.

MORISHITA, T. 1928 Comparative study of certain aciduric organisms of intestinal and dental origin. Proc. Soc. Exper. Biol. and Med., **25**, 654–656.

RAHE, A. H. 1915 A study of the so-called implantation of the *Bacillus bulgaricus*. Jour. Infect. Dis., **16**, 210–220.

RETTGER, L. F., AND CHEPLIN, H. A. 1921 The Transformation of the Intestinal Flora, with Special Reference to the Implantation of *Bacillus acidophilus*. Yale University Press.

RETTGER, L. F., AND HORTON, G. D. 1914 A comparative study of the intestinal flora of white rats kept on experimental and ordinary mixed diets. Centralbl. f. Bakteriol., 1 Abt., Orig., **73**, 362–372.

TORREY, JOHN C. 1915 The fecal flora of typhoid fever and its reaction to various diets. Jour. Infect. Dis., **16**, 72–108.

EFFECT OF HYDROGEN ION CONCENTRATION ON THE TOXICITY OF SODIUM BENZOATE TO MICROÖRGANISMS

W. V. CRUESS AND P. H. RICHERT

Fruit Products Laboratory, University of California

Received for publication November 30, 1928

Observations made several years ago in this laboratory indicated that foods of low acidity were much more difficult to preserve with sodium benzoate than those of high acidity. Thus, the spoilage of apple and grape juice by yeast and mold was prevented by 0.1 per cent or less of sodium benzoate, whereas 0.2 per cent failed to prevent growth of bacteria and mold in avocado pulp and in non-acid vegetables stored in weak brine.

It was suspected that hydrogen ion concentration rather than total acidity might be the controlling factor.

A study of the literature showed that the attention of previous investigators had been given to the effect of hydrogen ion concentration on the disinfecting, that is killing action, of sodium benzoate and various other reagents rather than to their preservative action.

However, Herter in 1910 reported that 0.2 per cent sodium benzoate retarded the growth of and gas production by *B. coli* in plain glucose bouillon, but had no noticeable effect in the same medium in the presence of $CaCO_3$. He made no pH determinations and gave the factor of acidity only passing attention.

Barnard (1911) states that benzoic acid is a more effective preservative than sodium benzoate but cites no experimental evidence. Held (1915) found that benzoic acid is less effective as a disinfectant in a medium rich in protein than in one poor in this constituent and states that if the protein binding power of the benzoic acid is satisfied by some other acid, such as tartaric, the concentration of benzoic acid necessary for disinfection is

lessened. Perry and Beal (1920) found that growth of *S. cerevisiae* in glucose bouillon was prevented by 0.5 per cent sodium benzoate and all living cells killed by 3.0 per cent. They also state that benzoic acid is more effective than sodium benzoate in preventing growth of yeasts and molds. Bonacorsi (1923) states that the disinfecting power of several common disinfectants is affected by the pH value of the medium but makes no statement concerning the effect of pH value on their inhibitive action on growth, or activity of microörganisms. Fleischer and Amster (1922) found that the disinfecting power of acid dyes is increased by a decrease in pH value and that of basic dyes by increase in pH value. Kuroda (1926) found that the killing action of sodium benzoate and several other organic preservatives and disinfectants was markedly affected by the pH value of the medium. Disinfection was much less effective in the pH range 5.0 to 8.9 than in that of 1.4 to 3.5. He used *B. coli* and *B. prodigiosus*.

EXPERIMENTAL

Our experiments were conducted for the purpose of definitely determining the effect of hydrogen ion concentration on the inhibiting action of sodium benzoate on the more common food spoilage microörganisms. Those studied were *Saccharomyces ellipsoideus*, isolated from grapes; *S. cerevisiae*; several Mycoderma yeasts; penicillium mold, two species; a mucor mold; a lactic acid culture from E. B. Fred (his culture No. 124-2) vinegar bacteria; *B. coli*, *B. sporogenes* and *B. subtilis*. It will be impossible to present all of the data in detail in this brief report; instead only the more significant results will be given and for the most part in the form of curves.

For the budding fungi, that is yeasts and molds and for vinegar bacteria, two fruit juice media, grape and apple juices, were used. Different portions were brought to various pH values by the addition of powdered citric acid or N/1 sodium hydroxide or in some instances sodium bicarbonate. These portions of various pH values were subdivided and sodium benzoate added in amounts ranging from no benzoate to an amount at each pH value that it was believed from preliminary tests would prevent growth.

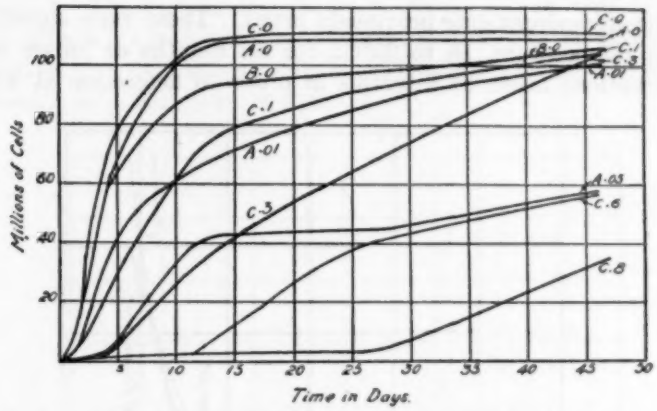

FIG. 1. EFFECT OF pH VALUE ON RETARDING ACTION OF SODIUM BENZOATE ON MULTIPLICATION OF S. ELLIPSOIDEUS

Curves labeled A 0, A 0.01 etc. represent juice of pH 3.8 and 0, 0.01 etc. gram benzoate per 100 cc. respectively; B, those of pH 3.0 and C those of pH 6.

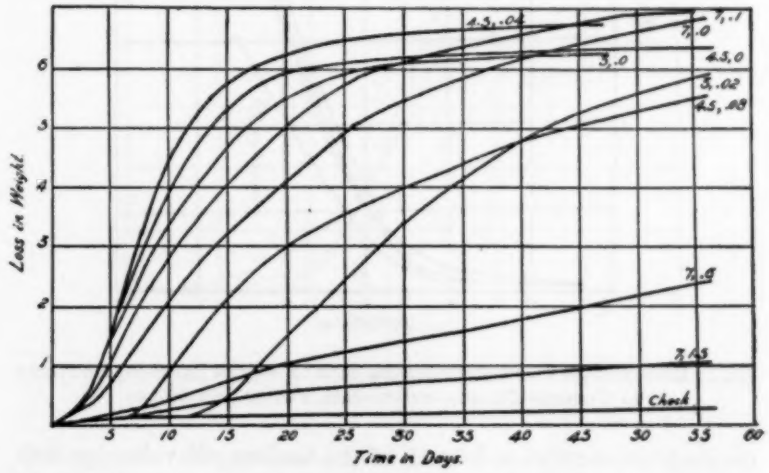

FIG. 2. EFFECT OF pH VALUE ON RETARDING ACTION OF SODIUM BENZOATE ON FERMENTATION BY S. ELLIPSOIDEUS

The tubed sterilized liquids were inoculated with pure cultures of the microörganisms previously listed. These were stored at room temperature, 18 to 25°C., for six months or longer and observations taken at intervals in order to determine at what

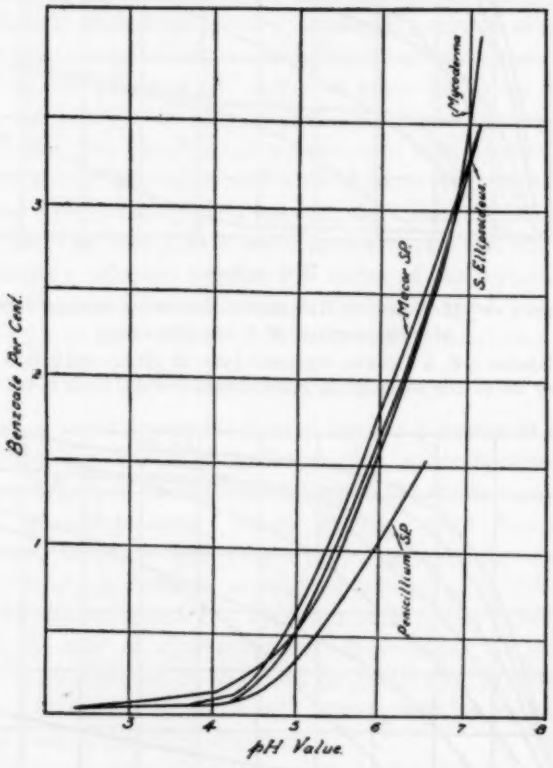

FIG. 3. EFFECT OF pH VALUE ON CONCENTRATION OF SODIUM BENZOATE REQUIRED TO PREVENT GROWTH OF CERTAIN YEASTS AND MOLDS

benzoate concentration in each of the various pH values growth was prevented.

With *S. ellipsoideus* additional observations were taken. In one series of tests the rate of multiplication of the cells at various

benzoate concentrations in juice of three pH values was determined. The results are shown in figure 1. In two other series of tests the rates of fermentation at three and at ten different pH values and various benzoate concentrations were determined. Typical results are given in figure 2.

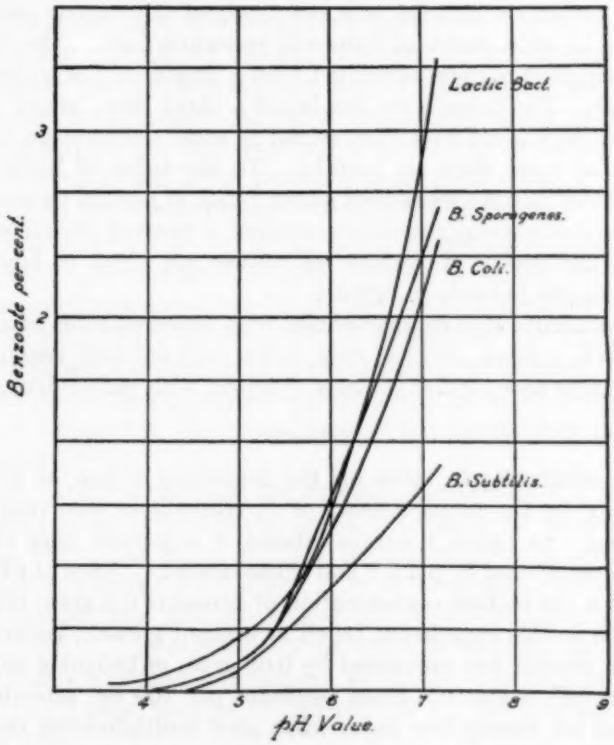

FIG. 4. EFFECT OF pH VALUE ON CONCENTRATION OF SODIUM BENZOATE REQUIRED TO PREVENT GROWTH OF CERTAIN BACTERIA

B. coli, *B. sporogenes*, *B. subtilis* and the lactic culture (E. B. Fred No. 124-2) were grown in a broth of the following composition; bacto pepton 10 grams, glucose 7.5 grams, Libby's extract of beef 10 grams, $MgSO_4$ 0.01 gram, KH_2PO_4 0.25 gram, $(NH_4)_2$

HPO_4 0.25 gram and water to make 1000 cc. The pH value of portions of the broth was adjusted by the addition of citric acid, N/1 NaOH or powdered $NaHCO_3$ to values ranging from 3.2 to 10.0 before sterilization. The range after sterilization was 3.6 to 8.7, changes occurring during sterilization being fairly pronounced at the lowest and highest pH values. Benzoate was added before sterilization to subdivisions of the various portions to give a wide range of benzoate concentrations. The tubed sterilized liquids were inoculated with a loopful of the respective cultures. The tubes were incubated a short time, about three days, at 37°C. and were then stored at room temperature 18° to 25°C. for more than six months. To the tubes of lactic and sporogenes cultures was added about ¾ inch of neutral mineral oil.

The benzoate concentrations required to prevent growth of the yeasts and molds at various pH values are given in figure 3; those for the bacteria in figure 4.

Experiments were conducted also with mixed cultures and with ripe olives in brine, avocado pulp, and certain non-acid vegetables. The results were similar to those obtained with pure cultures.

DISCUSSION

The effect of pH value on the inhibiting action of sodium benzoate on the multiplication of *S. ellipsoideus* was very pronounced. In figure 1 curves labeled A represent juice of pH 3.8, those labeled B, pH 3.0 and those labeled C, juice of pH 6.0. At pH 6 the highest concentration of benzoate 0.8 gram per 100 cc. used in this experiment failed to prevent growth, whereas at pH 3.0 growth was prevented by 0.05 gram of benzoate per 100 cc. At pH 6 and 0.8 gram benzoate per 100 cc. growth was delayed for twenty-five days, when slow multiplication ensued. At pH 6 and 0.3 gram benzoate per 100 cc., growth was slow but at 0.1 gram benzoate, growth was only moderately retarded. At the other pH values also, the retarding effect of the benzoate on growth varied with the concentration, although far less benzoate was required to produce a given effect at the lower pH values.

In figure 2 is given the effect of pH value on the retarding action of sodium benzoate on alcoholic fermentation by *S.*

ellipsoideus. As in its effect on the multiplication of this organism the retarding action of the benzoate was found to be dependent to a very marked degree on the pH value. The pH values used in this experiment were 3.0, 4.5 and 7.0. At pH 7.0 fermentation proceeded slowly even in the presence of 1.5 per cent sodium benzoate, the maximum concentration used. At pH 3.0 fermentation was completely prevented at 0.06 per cent of benzoate. At pH 4.5 more than 0.1 per cent of benzoate was required to prevent fermentation but 0.04 per cent appeared to stimulate fermentation. At benzoate concentrations between 0 per cent and the per cent required for prevention of fermentation the retarding action of the benzoate at each pH value in general varied with the concentration of benzoate present.

In figures 3 and 4 it will be seen that the benzoate concentration required to prevent growth varied greatly with the pH value. In the neighborhood of pH 4.5 for the yeasts and molds and at about 5.3 to 5.5 for *B. coli*, subtilis, sporogenes and lactic acid bacteria, there was great change in the slope of the curves. They became much steeper beyond these points; i.e. much more benzoate is required in the upper range of pH values than in that, say below pH 4.5.

Other data, not shown in the curves, indicate that the vinegar bacteria culture used behaved similarly to the lactic culture; and that at pH values 6.0 to about 9, growth of all organisms used was not prevented by 1.5 per cent of benzoate. At pH 10, less benzoate was required to prevent growth of one organism than at pH 7.3; in one test 0.7 per cent of benzoate prevented growth of one mycoderma culture at pH 10, but blue mold (a penicillium) grew even in the presence of 1.5 per cent benzoate at pH 10.0.

It is possible that the curves pass through a maximum somewhere between pH 7 and 10 and that the OH^- ion as well as the H^+ ion exerts an effect on the preservative action of sodium benzoate.

It is possible also that some of the inhibitive action of sodium benzoate at pH values in the range 5.0 to 7.0 is due to the Na^+ ion as Winslow (1928) and Doloff (see also the references given by

Winslow in article cited) show that sodium chloride at 0.5 M is slightly toxic in a pepton medium. At pH 7 in our tests concentrations of up to 4 per cent of sodium benzoate were used; giving at the higher percentages concentrations of Na^+ ions somewhat less than in a .5 M sodium chloride solution.

As would be expected, the pH growth range of the bacteria that are not tolerant to acid (*B. coli*, subtilis and sporogenes) was different from that for the acid tolerant organisms used in these tests and apparently also the turning point of the "pH versus benzoate to prevent growth" curves for these organisms was at a higher pH value than for the acid tolerant organisms. However, the pepton of the medium might have exerted some effect in the tests with these organisms.

It was found that ripe olives and non-acid vegetables in brine and avocado pulp inoculated with mixed cultures required many times more benzoate for preservation at pH values above 5 than at pH 3.5 to 4.0. One-tenth of 1 per cent benzoate had no apparent retarding effect on the spoilage of these products unless acid was added. It is therefore extremely dangerous to attempt to preserve such non-acid products with sodium benzoate unless acid is added. *B. subtilis* was considerably less tolerant to the acid and benzoate than *B. coli* and sporogenes.

SUMMARY

1. The retarding action of sodium benzoate on the rate of multiplication of *S. ellipsoideus* is much stronger at pH values of 2.5 to 4.5 than at 5.0 to 9.

2. A similar relationship holds for the retarding effect of sodium benzoate on alcoholic fermentation by this microörganism.

3. The concentrations of benzoate required to prevent the growth of *S. ellipsoideus*, *S. cerevisiae*, a mucor mold, two penicillium molds, three strains of mycoderma yeast, a lactic acid bacterium, a vinegar bacterium, *B. coli*, *B. subtilis* and *B. sporogenes* was greatly affected by the pH value of the medium. Much more benzoate was required at pH values near neutrality, e.g. pH 5 to 8 than at those in the moderately acid range 2.5 to 4.5.

4. While these observations are primarily of scientific interest

they also have an important bearing on the preservation of non-acid foods such as ripe olives, avocado pulp and non-acid vegetables by sodium benzoate.

5. Preliminary experiments have proved that similar relationships hold for certain other food preservatives.

REFERENCES

BARNARD, H. E. 1911 Sodium benzoate as a preservative in food. Chem. Eng., **12**, 104.

BONACORSI, L. 1923 Ueber den Einfluss der Reaktion des Nährbodens auf die entwicklungs hemmende Wirkung Chemischer Substanzen. Z. Hyg. Infekt., **99**, 284.

FLEISCHER, L., AND AMSTER, S. 1922 Ueber den Einfluss der Reaktion des Mediums auf die Disinfektions wirkung organischer Farbstoffe. Z. Immunitäts, **37**, 327.

HELD, D. 1915 The preserving action of benzoic acid. Arch. Hyg., **84**, 289.

HERTER, C. A. 1910 The action of sodium benzoate on the multiplication and gas production of various bacteria. J. Biol. Chem., **7**, 59–67.

KURODA, T. 1926 Ueber den Einfluss der Wasserstoffionen Konzentration auf die antiseptische Wirkung einiger Phenole und aromatischer Säuren. Biochem. Z., **169**, 281.

PERRY, M. C., AND BEAL, A. D. 1920 The quantities of preservatives necessary to inhibit and prevent alcoholic fermentation and the growth of molds. Jour. Ind. and Eng. Chem., **12**, 253.

WINSLOW, C.-E. A., AND DOLLOFF, A. F. 1928 Relative importance of additive and antagonistic effects of cations upon bacterial viability. Jour. Bact., **15**, 67–92. See also the references listed by these authors.

THE DESTRUCTION OF LUMINOUS BACTERIA BY HIGH FREQUENCY SOUND WAVES

E. NEWTON HARVEY AND ALFRED L. LOOMIS

From the Loomis Laboratory, Tuxedo Park, New York, and the Physiological Laboratory, Princeton University

Received for publication December 7, 1928

In a previous communication (1928) we have described the dimming of the light of luminous bacteria exposed to high power high frequency sound waves, generated by the apparatus of Wood and Loomis (1927). This consists of a 2-kilowatt oscillator giving a maximum of 50,000 volts across a quartz crystal in an oil bath. A test tube containing the bacteria suspended in sea water touches the surface of the oil, and the sound waves generated in the vibrating quartz crystal by the piezo-electric effect, pass through the oil, the glass of the test tube and the sea water suspension of the bacteria. Considerable energy is introduced, which heats the media, so that in the original experiments, using a frequency of 406,000, the bacteria were first cooled to 1.5° and rayed until their temperature reached 21.5°. This takes only a few minutes, but in this time the bacterial light is perceptibly dimmed, although not extinguished.

If a low powered oscillator is used and the bacteria mounted under a cover slip on the crystal and placed on the microscope stage without an oil bath, as described in our previous paper (1928), one can observe great agitation of the bacteria but no breaking up, such as happens to infusoria. The bacteria are carried into nodes of a complex wave pattern and remain unharmed, but the convection currents in a large test tube prevent the formation of permanent nodal accumulations of the bacteria.

In our recent experiments the bacteria (rod shaped *Bacillus Fisheri*)[1] were placed in a large test tube containing a glass coil

[1] I express sincere thanks to my students, Dr. S. E. Hill, Mr. C. S. Shoup and Mr. E. Holcomb for growing and supplying the luminous bacteria used in these experiments.—E. N. H.

through which ice water was circulating to keep down the temperature, and with another glass tube opening under the surface of the bacterial suspension, through which air, filtered through sterile cotton wool, was bubbled to keep the suspension stirred. The top of the test tube was cotton plugged and sterilized and the whole experiment so carried out as to prevent contamination with foreign bacteria. The high power oscillator was used with a frequency of 375,000 per second. It was found that even with ice water cooling of the suspension, the oil bath became so hot that in thirty minutes the temperature of the bacteria had risen to 35°C. As this is sufficient to cause near extinction of the light of the bacteria without exposure to sound waves, the oil bath in later experiments was also cooled with coils of lead tubing. The lead tubing and all metal parts near the high tension field must be grounded to prevent sparking.

After the exposure to sound waves the bacteria were inoculated on agar slants and Petri dishes to determine if all were killed. A number of experiments were carried out, as follows:

A1. Rayed for thirty minutes, when the suspension had almost completely cleared, an indication that the bacteria are broken up, and the light had disappeared, but this might be due to heating, as the temperature had risen to 35°C.

A2. Same procedure and results as A1.

A3. The same bacterial emulsion was heated to 35°C. in a water bath for thirty minutes and then cooled. It did not clear, but the bacterial luminescence had disappeared.

Platinum loop transfers of A1, A2, and A3, were made to agar slants as well as from a control suspension of bacteria unheated. After sixteen hours the control showed good luminescence and growth while A1, A2 and A3 showed no luminescence or growth. After forty-eight hours, A3, the heated tube, showed good luminescence from about one hundred colonies while A1 and A2 showed luminescence and growth from twelve and three colonies respectively. Evidently the heating had seriously injured the bacteria and the raying plus the heating had killed nearly all. There were no growing colonies without luminescence. This experiment indicates that the bacteria are mostly killed by one

half hour raying, but does not tell whether the luminescence can be completely extinguished, as the temperature rose too much.

In another experiment (B1) the bacteria were rayed for an hour and the temperature kept below 19°C. by cooling the oil as well as the bacterial suspension. The rayed bacteria showed no growth or luminescence when plated on the Petri dishes or when inoculated on agar slants, while the control inoculations grew and luminesced brilliantly. The luminescence dims rather quickly on raying but the last trace of light persists for something like twenty to thirty minutes.

Examined under the microscope the rayed suspensions show practically no bacteria while the controls are full of actively moving individuals. As indicated by the clearing of the suspensions the bacteria must be thoroughly torn up and cytolysed as they are when placed in water. The silkiness, due to optical effects of needle particles, observed when normal luminous bacteria suspended in sea water are gently agitated, had disappeared in the rayed tubes. Another experiment (B2), like the above, but in which the temperature was kept below 15°C., gave identical results.

C1. Finally a very dense emulsion of luminous bacteria in sea water was rayed for ninety minutes and loop samples inoculated on agar slants every five minutes. The temperature was kept below 16°C. throughout the experiment. The luminescence gradually dimmed until it was faint (forty-five minutes) and only a just perceptible luminescence remained after ninety minutes raying. After fourteen hours the inoculations made up to fifty minutes raying all showed good luminescent growth, the fifty-, sixty-, and seventy-minute inoculations a few colonies, and the eighty-, and ninety-minute inoculations no luminescence. After twenty-four hours all inoculations showed good luminescent growth except the ninety-minute with one colony and after thirty-six hours, all inoculations showed some luminescent growth. The raying had evidently killed most of the bacteria, only a few more resistant ones persisting with their power of growth retarded.

The rayed emulsion had partially cleared and lost most of its silky appearance on gentle shaking, characteristic of rod and

needle particles in suspension and examined under the microscope showed very few rods but many small granules, like fragments of bacteria. There is also a coagulum of fiber-like material which collects on the side of the test tube and cooling tubes, in all the rayed suspensions. This is not cotton fiber as it gives a strong xanthoproteic test and is undoubtedly composed of the debris of the bacteria stuck together in some peculiar manner under the influence of the sound waves. It never appeared in control unrayed tubes of bacterial suspension through which air was bubbled as in the experimental tubes.

It should again be emphasized that the conditions of the raying are such in these experiments, namely, a round bottomed tube and constant agitation, that no standing waves can be set up and consequently the bacteria cannot be thrown into nodes, where they remain unharmed, as happens in capillary tubes or spaces.

In order to see whether needle crystals could be broken up by raying, a suspension of benzopurpurin in water was rayed for one hour, with ice water cooling. The needle crystals, easily visible under high power of the microscope, are broken apart into shorter lengths but not sufficiently so as to abolish the silkiness normally exhibited by these solutions. A silky soap solution with needle crystals was not affected by one hour's raying. Perhaps we should not expect such needle particles whose consistency and rigidity undoubtedly differ from that of bacteria to behave in the same way.

In conclusion we can state that, under proper conditions of raying, luminous bacteria can be broken up and killed by sound waves of approximately 400,000 frequency and the solutions sterilized, but that the method is not one of any practical or commercial importance because of the expense of the process.

REFERENCES

HARVEY, E. N., AND LOOMIS, A. L. 1928 Nature, **121**, 622–624.
WOOD, R. W., AND LOOMIS, A. L. 1927 Phil. Mag., **4**, 417–436.

AN IMPROVED METHOD OF CULTURE FROM A SINGLE BACTERIAL CELL

SYDNEY G. PAINE AND J. C. RAMCHANDANI

Department of Plant Pathology, Imperial College of Science and Technology, London

Received for publication December 7, 1928

The method to be described is a modification of the one given by the senior writer in an earlier number of this JOURNAL (Paine, 1927) coupled with that of Burri (1909). Attenuations from the culture are made in five sterile watch glasses, as in the method of Paine but, in place of sterile water, use is made of a sterile solution of nigrosine as in the method of Burri. With the precautions described in the earlier paper the appropriate dilutions are taken up with a steel mapping-pen and, instead of being spotted on the surface of agar in a dish, are drawn in lines on the surface of a film of agar previously prepared on a long narrow coverslip. In order to prevent ploughing up the surface of the agar the steel pen is attached to its holder by a short coil of spring-brass wire. In this way sufficient rigidity for the control of the pen's direction can be retained while allowing only the slightest pressure of the point of the pen on the surface of the agar. The coverslip is mounted above a glass slide, film side down, conveniently supported at the ends on thin strips of cardboard in such a way that contact of the agar film with the glass slide is just prevented. The ink lines are examined under the 12th inch objective and the positions of what appear to be single bacterial cells are marked down by spots of Chinese ink. The slides are then placed in a moist chamber and incubated over-night. Any colonies which develop at the marked spots from single cells will be of approximately the same size and may safely be assumed to be pure, while any which develop into larger that minimal size may be discarded as impure.

REFERENCES

BURRI, R. 1909 Das Tuscheverfahren. Gustav Fischer, Jena.

PAINE, S. G. 1927 A practical method of culture from a single bacterial cell. Jour. Bact., 14, 441.

THE VALUE OF VEGETABLE EXTRACTS IN CULTURE MEDIUMS[1]

LUTHER THOMPSON

Section on Clinical Pathology, The Mayo Clinic, Rochester, Minnesota

Received for publication December 27, 1928

A survey of the literature dealing with the use of various extracts from vegetables and meats as enriching agents in culture mediums shows that certain workers (Davis, 1917 and 1921; Funk and Dubin, 1921; Hosoya and Kuroya, 1923a and b; Kligler, 1919; Lloyd, 1916–17; Thjötta, 1921; Thjötta and Avery, 1921 a and b; Uyei, 1927) have considered that they were dealing with vitamines or growth-accessory substances for bacteria. The organisms which are grown with more than average difficulty are the ones usually spoken of as requiring the presence of vitamines, such as *Diplococcus pneumoniae, Neisseria intracellularis, Neisseria gonorrhoeae, Hemophilus influenzae* and various streptococci. Pacini and Russell (1918) have shown that *Eberthella typhi*, when grown in a synthetic medium, will furnish vitamines which will maintain normal development in rats, but the work of others (Ayers and Mudge, 1922; McLeod and Wyon, 1921; Thjötta, 1924) indicates that the vitamines A and B, effective in animal nutrition, do not influence bacterial growth. Another group of investigators (Cole and Lloyd, 1916–17; Morgan and Avery, 1923; Mueller, 1922 a and b; Rivers and Poole, 1921) has called attention to the value of extracts of meat, blood and various vegetables in the culture of bacteria without suggesting anything regarding the nature of the growth-promoting substance.

Extracts were made of potato, carrot, radish, spinach and beef heart, the latter for comparison. One hundred fifty grams of grated or ground material were added to 200 cc. of distilled

[1] Read before the Society of American Bacteriologists, Richmond, Virginia, December 27 to 29, 1928.

water. The mixture was stirred for ten minutes and filtered through cheese cloth to remove the coarser particles. The filtrate was then centrifuged at high speed for thirty minutes and the supernatant fluid filtered through Seitz filters and stored in sterile test tubes in the ice box. Table 1 shows the total nitrogen and reducing sugars in these extracts, and indicates approximately what may be expected with this method of preparation, although there are variations between different lots.

When freshly made, the various extracts seemed to be about equal in growth-promoting power, but certain ones, especially those from spinach and beef heart, turned acid on standing and a precipitate was formed, after which their value was greatly decreased. The various extracts were heated to 56°C. for thirty

TABLE 1

EXTRACT	REDUCING SUGAR	TOTAL N
	per cent	*mgm. per cc.*
Potato	0.21	1.23
Carrot	0.86	0.42
Radish	0.36	0.18
Spinach	0.12	0.42
Beef heart	0.04	0.68

minutes, at 100°C. for ten minutes and run in the autoclave at 20 pounds pressure for fifteen minutes. After heating they were tested for growth-promoting power. In general it was found that heat had little effect except as it caused precipitation of the protein content. This was marked in some of the extracts. Potato extract was precipitated least and was found to be the best of those tried, so that most of the following work was done with it. This extract seems to keep for three to four months without losing effectiveness. In order to learn whether all the growth-giving material could be removed by precipitating the protein, a sample of potato extract was treated with 9 volumes of tungstic acid. The resulting precipitate was removed by centrifuging and the supernatant fluid was filtered to insure sterility. Tests showed that approximately half the total nitrogen was

proteid nitrogen. The precipitate was dissolved in weak alkali and made up to original volume. By using volumes of the two fractions which were equivalent to the original, it was found that both the proteid nitrogen and nonproteid nitrogen of potato extract would support equal growth of test organisms, but the growth was less than with the original. In other words, it was found that within certain limits, growth corresponded to the nitrogen content of the extract.

By the addition of increasing amounts of the extracts to tubes each containing 6 to 7 cc. of plain broth, which in this work was used as a routine basic medium, it was found that as little as 0.01 cc. would allow growth of many strains of streptococci which did not grow in the basic medium. An increase in the

TABLE 2

	TUBE 1*	TUBE 2*	TUBE 3*	TUBE 4*	TUBE 5*	TUBE 6*	TUBE 7*	TUBE 8*	TUBE 9*
Potato extract added, cc.	0.01	0.02	0.03	0.05	0.1	0.2	0.5	1.0	None
Growth in twenty-four hours	+	++	++	+++	+++	+++	+++	+++	None

* Each with 6 cc. of broth.

luxuriance of growth of streptococci was not noted when amounts greater than 0.2 cc. were added to the same amount of medium. Table 2 shows a typical result.

After the addition of the extract all tubes were incubated for twenty-four hours as a proof of sterility before they were inoculated. Then one capillary drop of a light suspension of hemolytic streptococcus in physiologic sodium chloride solution was added to each tube.

The value of potato extract in promoting growth of streptococci was tested as follows: To tubes containing 6 to 7 cc. of nutrient broth was added 0.2 cc. of the extract. The streptococci were picked from blood agar plates and were kept as stock cultures on blood agar slants. From these stock cultures a small amount was transferred to the test medium with a straight wire, care being taken not to carry over any of the medium. Nineteen

cultures of hemolytic streptococci grew freely within twenty-four hours. Four cultures of *Streptococcus viridans* were tested, three from the blood in cases of subacute bacterial endocarditis and one from chest fluid. All grew well in twenty-four hours, with the exception of one of the strains from endocarditis, which showed fair growth in forty-eight hours.

One culture of a hemophilic organism, corresponding culturally and morphologically with *Hemophilus influenzae*, did not grow in nutrient broth and potato extract, but did grow in brain broth and potato extract. Brain broth alone did not support growth of this organism.

Potato extract, when added to blood agar, has given a somewhat more vigorous growth of *Neisseria gonorrhoeae* than was obtained without it, but the results are not as striking as with the other types of organisms mentioned.

Certain experiments have been made to test the suitability of potato extract as an enriching substance in sugar fermentation tubes by adding 0.1 cc. of the extract to each tube containing 5 cc. of the sugar broth used (nutrient broth and 0.5 per cent sugar). The typical fermentation reactions of *Eberthella typhi* were unchanged by the addition of the extract. As a further test two strains of streptococci were chosen, one *Streptococcus viridans* which grew in the sugar broths without the addition of any enriching fluid, and the other a hemolytic streptococcus which would not. With each organism two series of tubes were set up, the first series with the extract added and the second series without it. The *Streptococcus viridans* fermented identical sugars in each series, thus proving that the addition of extract did not affect the results. The hemolytic streptococcus fermented certain of the sugars in the series of tubes containing the potato extract and failed to grow in the other series.

Attempts to grow *Diplococcus pneumoniae* in nutrient broth and potato extract have shown that growth may be obtained by using more of the extract than seems necessary with streptococci. The growth so obtained in two instances was used as an antigen in the typing test with clear-cut results, while the brain broth

cultures did not agglutinate. Further attempts are being made to determine the value of potato extract for this purpose.

The growth-promoting substance in vegetable extracts has been called a vitamine or growth-accessory factor by most workers. As previously stated, some have found that vitamine B does not aid the growth of bacteria, and have suggested that a special vitamine is necessary for bacteria. It is difficult to devise experiments which will show conclusively that a substance of unknown composition is or is not a vitamine without resorting to well established experiments in animal nutrition. I believe that the substance in fresh plant and animal tissues, which are water-soluble, may be better considered as food substances for bacteria than as food-accessory factors or vitamines. The following experiments and observations are made in support of this statement.

A tube of potato extract was used as a medium for growing *Salmonella schottmülleri*. After seven days' incubation the culture was heated to 80°C. for ten minutes and centrifuged at high speed to throw down the bacteria. The clear supernatant fluid was added to tubes of plain broth in varying amounts up to 0.2 cc. After these tubes were incubated to prove sterility they were inoculated with a culture of hemolytic streptococcus which was known to grow well when 0.2 cc. of potato extract was used. Growth was not obtained after the addition of the extract which had been used as a medium for *Salmonella schottmülleri*. Evidently the growth-promoting material had been exhausted by the paratyphoid organism. It is not surprising that a medium containing such small amounts of nitrogenous and carbohydrate substances should be exhausted by an actively growing organism in seven days. This experiment indicates that the growth-promoting substance is a food rather than a food-accessory factor, and it also shows that the growth of *Salmonella schottmülleri* of itself did not produce a food-accessory factor for the streptococcus used.

When the foregoing experiment was repeated with one change only, that is, the substitution of plain broth for potato extract, growth of the streptococcus was obtained. The paratyphoid

organism evidently does not use up all of the nitrogenous material when as much as 1 per cent peptone is supplied, but it does exhaust the nitrogenous material in potato extract if only 1 to 2 mgm. for each cubic centimeter is present. As a further test a plain broth containing 0.1 per cent peptone was used instead of the usual 1 per cent and the experiment was repeated. It was found that, with the smaller amount of peptone, growth of the streptococcus did not occur when the supernatant fluid of the paratyphoid culture was added to plain broth as before. To make sure that this was not a chance result the experiment was repeated several times, using six different Gram-negative bacilli for the treatment of the 1 and 0.1 per cent peptone broths. The cultures so obtained were tested for power to stimulate growth of ten different cultures of streptococci, with the results mentioned. It is understood, of course, that the streptococci used did not grow perceptibly in nutrient broth with the rather light inoculations employed. From these experiments it appears that, when a larger amount of nitrogenous material is used, the growth of the Gram-negative bacilli comes to an end before the food is exhausted and that the residual end-products furnish a suitable pabulum for the growth of streptococci. But when a small amount of nitrogenous material is employed, as in 0.1 per cent peptone or in potato extract, the residual material is not sufficient. This is taken as evidence that the significant factor in accelerating growth of streptococci is proper food rather than the presence of food-accessory factors.

In regard to the effect of the carbohydrate in the various extracts used, it was noted that there was no correlation between the amount of reducing sugar present and the power to stimulate growth. Extract of beef heart which contained only 0.04 per cent was slightly superior to carrot extract which contained twenty times that amount of reducing sugar.

SUMMARY

Aqueous extracts of potato, carrot, spinach, radish and beef heart were sterilized by filtration and added to nutrient broth aseptically. It was found that as little as 0.01 cc. of these

extracts, when added to 6 to 7 cc. of nutrient broth, would promote growth of many streptococci which did not grow in the broth alone, while 0.2 cc. was sufficient to give vigorous growth of most streptococci. Potato extract was found to be the most satisfactory, because of its high nutritive value and because it keeps well without change in reaction or precipitation of protein.

Heat was found to have an effect on the extract which was proportional to the precipitation, those media giving the most precipitate being the least useful in accelerating growth.

Both the proteid and nonproteid nitrogen fractions of the potato extract serve to stimulate growth of streptococci, but not in as marked a degree as the whole extract.

Potato extract may be used in place of fresh blood in many instances where it is not essential to observe hemolysis. It is helpful in getting a growth of streptococci and pneumococci free from cells and precipitated fractions of medium. As an enriching substance in ordinary fermentation tubes it allows growth of streptococci without interfering with the action on the sugar.

The substance in potato extract responsible for promoting growth is thought to be nitrogenous material which furnishes suitable food for the bacteria rather than food-accessory substances.

REFERENCES

Ayers, S. H., and Mudge, C. S. 1922 The relation of vitamines to the growth of a streptococcus. Jour. Bacteriol., **7**, 449–464.

Cole, S. W., and Lloyd, Dorothy J. 1916–1917 The preparation of solid and liquid media for the cultivation of the gonococcus. Jour. Path. and Bacteriol., **21**, 267–286.

Davis, D. J. 1917 Food accessory factors (vitamins) in bacterial culture with especial reference to hemophilic bacilli. I. Jour. Infect. Dis., **21**, 392–403.

Davis, D. J. 1921 Food accessory factors in bacterial growth. III. Further observations on the growth of Pfeiffer's bacillus (*B. influenzae*). Jour. Infect. Dis., **29**, 171–177.

Funk, C., and Dubin, H. E. 1921 Vitamine requirements of certain yeasts and bacteria. Jour. Biol. Chem., **48**, 437–443.

Hosoya, S., and Kuroya, M. 1923a Water-soluble vitamine and bacterial growth. First report. Scientific Reports from the Gov. Instit. for Infect. Dis., Tokyo Imperial Univ., **2**, 233–264.

HOSOYA, S., AND KUROYA, M. 1923b Water-soluble vitamine and bacterial growth. Second report. "With special reference to the chemical and physical properties of vitamine essential for growth of hemolytic streptococci." Scientific Reports from Gov. Instit. for Infect. Dis., Tokoyo Imperial Univ., **2**, 265–285.

KLIGLER, I. J. 1919 Growth accessory substances for pathogenic bacteria in animal tissues. Jour. Exper. Med., **30**, 31–44.

LLOYD, DOROTHY J. 1916–1917 On vitamines, amino-acids, and other chemical factors involved in the growth of the meningococcus. Jour. Path. and Bacteriol., **21**, 113–129.

M'LEOD, J. W., AND WYON, G. A. 1921 The supposed importance of vitamins in promoting bacterial growth. Jour. Path. and Bacteriol., **24**, 205–210.

MORGAN, H. J., AND AVERY, O. T. 1923 Studies on bacterial nutrition. IV. Effect of plant tissue on growth of pneumococcus and streptococcus. Jour. Exper. Med., **38**, 207–217.

MUELLER, J. H. 1922a Studies on cultural requirements of bacteria. I. Jour. Bacteriol., **7**, 309–324.

MUELLER, J. H. 1922b Studies on cultural requirements of bacteria. II. Jour. Bacteriol., **7**, 325–338.

PACINI, A. J. P., AND RUSSELL, D. W. 1918 The presence of a growth-producing substance in cultures of typhoid bacilli. Jour. Biol. Chem., **34**, 43–49.

RIVERS, T. M., AND POOLE, A. K. 1921 Growth requirements of influenza bacilli. Johns Hopkins Hosp. Bull., **32**, 202–204.

THJÖTTA, T. 1921 Studies on bacterial nutrition. I. Growth of *Bacillus influenzae* in hemoglobin-free media. Jour. Exper. Med., **33**, 763–771.

THJÖTTA, T. 1924 On the nature of the heat-labile substance ("V") required for the growth of the bacillus of Pfeiffer. Jour. Exper. Med., **40**, 671–678.

THJÖTTA, T., AND AVERY, O. T. 1921a Studies on bacterial nutrition. II. Growth accessory substances in the cultivation of hemophilic bacilli. Jour. Exper. Med., **34**. 97–114.

THJÖTTA, T. AND AVERY, O. T. 1921b Studies on bacterial nutrition. III. Plant tissues, as a source of growth accessory substances in the cultivation of *Bacillus influenzae*. Jour. Exper. Med., **34**, 455–466.

UYEI, N. 1927 Food accessory substance (vitamins) and tubercle bacilli. 2. The effect of the commonly known vitamins on the growth of tubercle bacilli. Jour. Infect. Dis., **40**, 433–437.